INFLUENCE OF INSULIN-LIKE GROWTH FACTOR-1, STEROIDS, AND NITRATE ON REPRODUCTION IN AMPHIBIANS

By

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Abstract of Dissertation Presented to the Graduate School
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Chair: Louis J. Guillette
Major Department: Zoology

My goal was to examine the influence of insulin-like growth factor-1 (IGF-1), 17-β estradiol (E_2), testosterone (T), and nitrate exposure on various aspects of reproduction in frogs. To accomplish this, I investigated seasonal changes in plasma IGF-1, E_2, and T concentrations in a wild population of *Rana grylio*. I also determined the importance of steroid and growth factor hormones in reproductive physiology by examining ovariectomized *Rana catesbeiana* for changes in plasma IGF-1, E_2, and T concentrations, and changes in oviduct morphology after treatment with known doses of IGF-1, E_2, and epidermal growth factor (EGF). Finally, I examined three aquatic frogs species (*Xenopus laevis*, *R. grylio*, and *R. catesbeiana*) for the effects of nitrate exposure on changes in plasma IGF-1, E_2, and T concentrations, and on oviduct morphology.

I have demonstrated that plasma IGF-1, E_2, and T concentrations (and reproductive tissue growth) exhibit a clear seasonal pattern of changes that overlap with changes in environmental variables, such that reproductive condition is optimized to match favorable environmental temperatures. I also demonstrated that E_2 is a potent stimulator of oviduct growth, while EGF and

IGF-1 do not induce oviductal growth in *R. catesbeiana*. I also provide the first evidence that exposure to environmentally relevant concentrations of nitrate alters endocrine hormones in *Xenopus laevis*, *R. grylio*, and *R. catesbeiana*. Furthermore, IGF-1 and steroid hormone concentrations are altered with exposure to nitrate at concentrations deemed safe for human drinking water by the US EPA (10 mg/L). *In vivo* exposure of *X. laevis* (for 7 continuous days) to nitrate concentrations below 50 mg/L significantly increased plasma IGF-1 concentrations, and inhibited ovarian E_2 and T synthesis. *In vitro* incubation of ovarian tissue (from wild-caught *R. grylio*) with nitrate concentrations between 0.17 and 33.00 mg/L nitrate (and between 0.20 and 40.60 mg/L nitrite) inhibited E_2 and T synthesis after 3 hours of exposure. Lastly, *in vivo* exposure of *R. catesbeiana* to nitrate concentrations between 1.65 and 16.50 mg/L increased plasma IGF-1, E_2, and T concentrations; and caused oviductal atrophy. These findings demonstrate that exposure to nitrate at extremely low concentrations causes endocrine disruption in frogs.

CHAPTER 1
INTRODUCTION: INFLUENCE OF STEROIDS, INSULIN-LIKE GROWTH FACTOR-1, AND AQUATIC NITRATE ON REPRODUCTION IN AMPHIBIANS

Reproductive Steroids and Amphibian Reproduction

Amphibians display some of the most diverse reproductive modes compared to other vertebrates. Most amphibians exhibit the ancestral reproductive mode, and are restricted to water to oviposit and fertilize eggs externally. Some species are terrestrial breeders, have internal fertilization, and either oviposit eggs on land or retain them within the oviducts for all or part of the embryonic developmental period. Finally, some amphibians oviposit terrestrial eggs from which offspring hatch, bypass the free-living tadpole stage, and undergo direct development to emerge as fully developed froglets (Wake and Dickie, 1998).

The reproductive system in amphibians is characterized by cyclic changes in growth and function that are modulated by hypothalamic releasing hormones, pituitary gonadotropins, and gonadal steroids. The process of steroid synthesis or steroidogenesis is regulated primarily by the hypothalamic-pituitary-gonadal axis (Licht, 1970, 1979; Licht et al., 1983). Gonadotropin-releasing hormone (GnRH) is secreted by the hypothalamus (in response to internal or environmental cues) and stimulates the anterior pituitary to release luteinizing hormone (LH) and follicle stimulating hormone (FSH) into the bloodstream. These gonadotropins stimulate gonadal steroidogenesis and gametogenesis. The principal gonadal steroids are progesterone (P_4), estradiol 17β (E_2), and testosterone (T). Theca interna cells within the ovary synthesize T in response to LH stimulation. In response to FSH stimulation, ovarian granulosa cells synthesize aromatase,

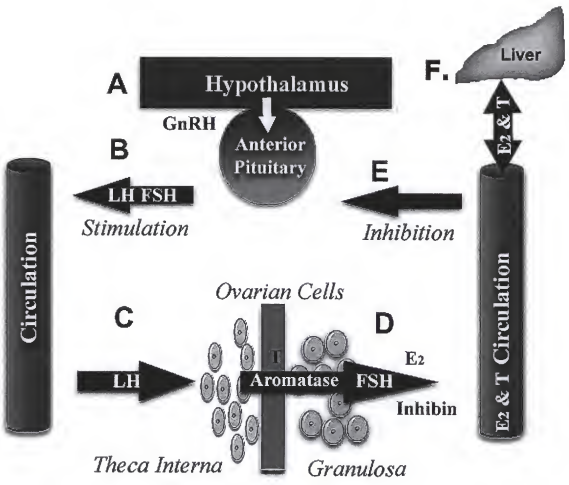

Figure 1-1. Regulation of gonadal steroidogenesis. (A) Hypothalamic gonadotropin releasing hormone (GnRH) induces pituitary secretion of luteinizing hormone (LH) and follicle stimulating hormone (FSH) (B). LH stimulates theca interna cells (C) to synthesize testosterone (T). FSH stimulates granulosa cell to produce aromatase enzymes (D), which convert T into estrogen (E_2). Steroidogenesis induces inhibin release from the gonad, which inhibits further hypothalamic and pituitary stimulation (E). F. Circulating steroids are also metabolized and cleared from the bloodstream by the liver.

an enzyme that converts T into E_2 (Figure 1-1). These steroids induce gonadal release of inhibin, a hormone that inhibits hypothalamic-pituitary stimulation of further steroid synthesis. Gonadal steroids exert an autocrine or paracrine action, by influencing localized tissues; or function as endocrine hormones when released into the bloodstream, to affect distant target tissues. Within steroid-responsive tissues, T and E_2 bind to and activate cytosolic or nuclear receptors and form a steroid-receptor complex. This complex binds to a hormone-response element on DNA to

stimulate or inhibit transcription, protein synthesis, and tissue growth (Segars and Driggers, 2002). Through this process, E_2 and T regulate normal development of secondary sexual characteristics, regulate growth of steroid-responsive tissues, and regulate reproductive function (Guidice, 1999).

In amphibians, E_2 is essential for oocyte development and maturation within ovarian follicles (Dumont, 1971; Fortune, 1983). Gonadal E_2 and T regulate many aspects of reproductive function, such as oviduct growth and secretions (Licht et al., 1983; Norris, 1997). The oviduct is a vital structure for reproductive function in oviparous vertebrates, including amphibians (Giudice, 1992; Wake and Dickie, 1998). After ovulation from the ovaries, mature oocytes travel through the oviduct to the cloaca and are expelled into the environment. In addition to providing physical transport, the oviduct synthesizes and secretes proteins and other substances that nourish and encapsulate the ova, and also aid in fertilization (Low et al., 1976; Buhi et al., 1997).

Insulin-Like Growth Factor-1

It has become increasingly apparent that reproductive function and physiology are regulated by steroid-signaling pathways, and also by other pathways involving insulin-like growth factor-1 (IGF-1). Originally called somatomedin C, IGF-1 is a polypeptide hormone that is structurally similar to IGF-II and proinsulin, and likely originated early in vertebrate evolution. IGF-1 is part of the growth factor system, which consists of a family of proteins that function in regulating many cellular processes (including cell proliferation, differentiation, and apoptosis) in virtually all tissues. (LeRoith et al., 2001a,b). Thus, IGF-1 is important for normal growth and function of reproductive tissues, and also for somatic tissues. Accordingly, the role of IGF-1 in growth of reproductive and somatic tissues has been examined in a variety of vertebrates including mammals, fish, birds, and reptiles (Girbau et al., 1987; Murphy and Ghahary, 1990; De Pablo et al., 1990; Serrano et al., 1990; Simmen et al., 1990; Scavo, 1991; Kapur et al., 1992; Cox and Guillette, 1993; Tang et al., 1994; Guillette et al., 1996; Buhi et al., 2000; Qu et al., 2000; Allan et al., 2001). Although IGF-1 has been identified in the plasma and tissues of some

amphibians, the role of this peptide hormone in tissue growth and function in these animals remains unclear, and requires further study (Daughaday et al., 1985; Pancak-Roessler and Lee, 1990).

Traditionally, IGF-1 was thought to influence tissue growth primarily by mediating the effects of growth hormone (GH). This physiological function of IGF-1 is the basis of the original somatomedin hypothesis (LeRoith et al., 2001b). More recently, IGF-1 has been found to play an important role in growth and differentiation of reproductive tissues (independent of GH), by mediating the mitogenic effects of E_2 (Girbau et al., 1987; Murphy and Ghahary, 1990; Cox, 1994).

In the presence of E_2, IGF-1 has been shown to mediate growth of E_2-sensitive reproductive tissues like the oviduct (Mead et al., 1981; Murphy and Murphy, 1994). Research indicates that the growth effects of IGF-1 does not require E_2 but requires only the presence of the 17β estradiol alpha receptor (ERα) in reproductive tissues (Klotz et al., 2000). This is supported by findings of an E_2-like growth response in the oviduct of ovariectomized animals treated with IGF-1 (Cox, 1994). These findings demonstrate that IGF-1 potentiates E_2-induced growth and also stimulates E_2-independent tissue growth.

In addition to mediating the growth effects of reproductive steroids, IGF-1 has also been shown to regulate intraovarian steroid synthesis in mammals (Adashi et al., 1991; Guidice, 1992; Adashi, 1993). Decreased E_2 expression increases ovarian IGF-1 expression. Ovarian IGF-1 stimulates synthesis of E_2 and P_4, and increases aromatization of androgens into E_2 (Adashi et al., 1991). Additionally, the ovaries and oviduct synthesize and secrete IGF-1 in response to GH, FSH, E_2, and other hormones. Based on these findings, the list of factors that regulate (or are influenced by) the IGF-1 system has been expanded to include reproductive steroids.

Research spanning nearly 50 years has defined many components of the surprisingly complex IGF-1 system (Le Roith et al., 2001b). In all vertebrates examined, the liver synthesizes and secretes most of the circulating concentrations of IGF-1. Hypothalamic release of growth

hormone-releasing hormone (GHRH) stimulates the pituitary to secrete growth hormone (GH) into the bloodstream. In response to GH stimulation, the liver synthesizes and secretes IGF-1 into the bloodstream. Hepatic IGF-1 can affect peripheral tissues in a paracrine or autocrine manner; or it can be transported through the bloodstream, bound to IGF binding proteins (IGFBPs), as an endocrine hormone that mediates growth and apoptosis of distant target tissues. After reaching its target tissue, IGF-1 interacts with a transmembrane cell-surface IGF-1 receptor (IGF-1R) where it is released from its binding protein to initiate a cellular response. Excess circulating IGF-1 is then filtered and degraded by the kidneys (LeRoith et al., 2003). In this manner, IGF-1 mediates GH-induced cellular proliferation. This endocrine-signaling pathway is the basis of the original somatomedin hypothesis. However, recent research suggests that the somatomedin hypothesis should be revised. IGF-1 has been shown to have many GH-independent effects on regulating tissue growth. Additionally, non-hepatic tissues (including the ovaries and oviduct) are now known to synthesize and secrete IGF-1 (LeRoith et al., 2001b).

The extracellular functional components of the IGF-1 system include IGF-1, IGFBPs, and IGF-1R. Expression of IGF-1 can be stimulated by various factors including growth hormone, E_2, T, P_4, FSH, glucose, insulin, and thyrotropin; whereas, IGF-1 expression can be inhibited by somatostatin, LH, cortisol, and interferon. Six known IGFBPs can bind with IGF-1 to modulate cellular effects. The IGFBPs that regulate the cellular effects of IGF-1 include IGFBPs 1, 3, 4, and 5. The other binding proteins (IGFBPs 2 and 6) specifically regulate the effects of IGF-2 on embryonic development. The IGFBPs prevent IGF-1 degradation during circulation, transport IGF-1 to target tissues, and regulate binding of IGF-1 to IGF-1R. Like IGF-1, IGFBPs can be stimulated or inhibited by various factors. Another functional component of the IGF-1 system is the IGF-1R. The IGF-1R is a tyrosine kinase, transmembrane receptor found on virtually every tissue type, and it mediates a majority of IGF-1 actions on cell growth. There is an IGF-2 receptor, but it is highly specific for IGF-2 and functions mostly in mediating embryonic development. Expression of the IGF-1R can be stimulated by a variety of factors including E_2,

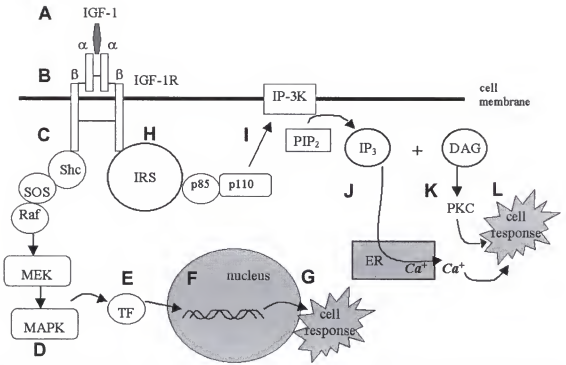

Figure 1-2. Binding of IGF-1 with the IGF-1R, initiates phosphorylation of intracellular proteins in a signaling cascade that leads to a cellular response. Activation of adaptor proteins includes the mitogen activated protein kinase (MAPK) pathway, the phosphatidylinositol 3-kinase (IP-3K) pathway and its secondary messengers IP_3, DAG, and Ca^+. Briefly, binding of IGF-1 (A) to the receptor (B) results in autophosphorylation of the intracellular β-subunit of the receptor (C). This then activates intracellular adaptor proteins, insulin receptor substrate (IRS) and Shc, to bind with the receptor and become phosphorylated. If adaptor protein Shc is activated, it forms a complex with SOS to activate Raf. Activation of Raf phosphorylates protein kinase MEK and leads to phosphorylation (D) of mitogen-activated protein kinase (MAPK). This activates transcription factors (TF) that bind to nuclear DNA (F) to elicit a cellular response (G). If the adaptor protein IRS is activated (H), a sequence of phosphorylations involving protein subunits p85 and p110 will activate the IP-3K pathway (I). Activation of IP-3K pathway phosphorylates the conversion of phosphoinositol bisphosphate (PIP_2) to the second messengers (J) inositol trisphosphate (IP_3) and (K) diacyglycerol (DAG). Each of these second messengers can induce cellular responses (L) either by activating the Ca^+/calmodulin complex by IP_3 or by the activation of phosphokinase C (PKC) by DAG.

FSH, LH, and oncogenes. Conversely, IGF-1, P4, and tumor suppressors can inhibit expression of the IGF-1R.

The functional IGF-1 system also has intracellular functional components that become activated by binding of IGF-1 to the IGF-1R. Once bound, the IGF-1R becomes phosphorylated, and a variety of intracellular proteins and second messengers are involved in a signaling cascade

that leads to a cellular response (Fig 1-2). Thus, each of the functional components of the IGF-1 system can be regulated by complex extracellular and intracellular factors.

Aquatic Nitrate and Amphibian Reproduction

In the past few years, there has been increased global concern over contamination of water by anthropogenic sources of nitrate. Nitrate is an anionic form of nitrogen that infiltrates watersheds in agricultural and urban environments, and reaches harmful concentrations largely due to human activities. In agricultural areas, watersheds are polluted with nitrate from unregulated run-off of nitrogen-based fertilizers and run-off of animal wastes. In urban areas, nitrates contaminate watersheds primarily through runoff of industrial and wastewater effluent from treatment plants and of fertilizers applied to lawns and golf courses (Rouse et al., 1999). The application of fertilizers in close proximity to watersheds during the spring frequently results in an overwhelming nitrate "pulse" that overlaps the breeding season of many amphibians. Unfortunately, most studies of the effects of nitrate on amphibians report nitrate concentrations differently, making comparisons and interpretation of these studies extremely difficult. For consistency throughout this dissertation, nitrate is reported as equivalent to nitrate-as-nitrogen (NO_3-N). This represents the concentration of nitrogen present in a given concentration of nitrate. Additionally, equivalent measures of nitrate are provided here to facilitate comparison among other nitrate and nitrite studies (Table 1-1).

Most studies on the effects of nitrate on amphibians have addressed toxicological rather than sublethal concentrations (Rouse et al., 1999). Most of these studies also focused on juvenile amphibian stages rather than on adults (Table 1-2). The impact of nitrate exposure on mammalian steroidogenesis has been examined and described in a few studies. Nitrate exposure has been shown to inhibit androgen synthesis in rodents *in vivo* and also in Mouse Leydig tumor cells *in vitro* (Panesar, 1999; Panesar and Chan, 2000). One mechanism for altered steroid expression (*in vivo*) by nitrates involves enzyme-dependent synthesis of nitric oxide (NO) (Panesar and Chan, 2000). The NO is synthesized from an L-arginine precursor by nitric oxide synthase (NOS)

enzymes (Kleinert et al., 1995; Mayer and Hemmenns, 1997). In addition to NOS-dependent NO formation, non-enzymatic synthesis of NO can also occur through acidic reduction of nitrite (Iizuka et al., 1999; Zweier et al., 1995, 1999; Modin et al., 2001). Cosby et al. (2003) reported that hemoglobin functions as a nitrite reductase contributing to enzyme-independent NO synthesis. Furthermore, Zweier et al., (1999) reported that enzyme-independent NO formation is associated with cellular damage and loss of organ function. Regardless of the mechanisms by which it is produced, NO is thought to regulate many physiological processes. Within the gonad, NO can inhibit steroidogenesis by binding to the heme (iron-containing) groups located on the enzymes of the cytochrome P450 superfamily necessary for steroid synthesis, like 3β-dehydroxysteroid dehydrogenase (3β-HSD). (Van Voorhis et al., 1994; Panesar and Chan, 2000). The IGF-1 counteracts the effects of NO by increasing ovarian E_2 synthesis (Van Voorhis et al., 1994; Van Voorhis et al., 1995; Srivastava et al., 1998; Inigues et al., 2001; Les Dees et al., 2001). Within the mitochondria of cells, the enzymes $P450_{scc}$ and 3β-HSD convert free cholesterol into P_4 (the precursor for T and E_2). Steroid enzyme pathways disrupted by NO can inhibit P_4 and downstream androgen synthesis (Panesar and Chan, 2000). If P_4 and T synthesis are inhibited by nitrate, then less androgen is available for aromatase enzymes to synthesize into E_2, and estrogen concentrations would be altered. Despite findings of endocrine disruption by nitrate in mammals, no study has examined whether nitrate disrupts endocrine function in adult, reproductive amphibians.

Since E_2 and IGF-1 interact to regulate growth-related responses in reproductive tissues, it is plausible that alteration of E_2 expression by nitrates might also influence IGF-1 and oviduct growth (perhaps through a NO-dependent pathway). In humans, intraovarian IGF-1 expression increases in response to elevated intraovarian NO. The IGF-1 apparently counteracts the inhibitory effects of NO on steroids, by stimulating increased expression of aromatase enzymes, StAR protein, P_4, and E_2. Thus, increased intraovarian IGF-1 in response to NO might be a

compensatory response, functioning to amplify steroid synthesis that has been compromised (Schams et al., 1988; Erickson et al., 1989; Adashi, 1993; Samaras et al., 1996; Iniguez et al., 2001; Les Dees et al., 2001). These studies indicate that IGF-1 plays a vital role in steroid synthesis and regulation, and possibly functions through an NO-dependent pathway. The mechanism by which steroids and IGF-1 interact to stimulate growth of reproductive tissue remains enigmatic and requires further study. In addition, the influence of nitrate exposure on reproductive physiology of anurans remains unknown.

Organic nitrate and nitrite are normally present in aquatic habitats, in low concentrations, due to bacterial breakdown of organic matter and accumulation of biological wastes. In addition to contributions from natural sources, anthropogenic sources of nitrate and nitrite can compromise water quality even further. Unusually high concentrations of nitrate and nitrite can accumulate in aquatic habitats that receive runoff of agricultural fertilizers and animal wastes. Aquatic nitrate and nitrite contamination might provide a biological signal to frogs that water quality is unsuitable for reproduction. High nitrate and nitrite concentrations might repress physiological changes that stimulate reproductive condition of frogs. Contamination of aquatic habitats with nitrate and nitrite has been shown to be detrimental to survival of anuran eggs and tadpoles (Table 1-2), and amphibian populations are reportedly declining in some agricultural areas (Berger, 1989).

Research Objectives

One goal of my study was to gain a better understanding of the interaction of IGF-1 with E_2-dependent and independent growth of reproductive tissues in aquatic amphibians. Although IGF-1 is important for cell growth and differentiation, abnormally high concentrations of plasma IGF-1 are associated with abnormal growth of reproductive tissues; and with cancer of the breast, ovaries, uterus, endometrium, and prostate (LeRoith et al., 1995a,b; Grimberg and Cohen, 1999; van Dessel et al., 1999; Werner and Le Roith, 2000; Smith et al., 2000). The IGF-1 and IGF-1R can protect cells from apoptosis; but in some mammals, over-expression of these receptors

induces ligand-dependent tumor formation. Over-expression of IGF-1R can be induced by up-regulation of IGF-1 expression in response to growth hormone (GH)-, E_2-, and ERα-dependent pathways (Kaleko et al., 1990). Additionally, uterine IGF-1 and IGF-1R up-regulation (along with increased uterine epithelial cell growth) occurs in ovariectomized rodents in response to synthetic estrogens (DES and bisphenol A) and phytoestrogens (Klotz et al., 2000). From these findings, I hypothesized that endocrine disrupting contaminants (EDCs) could affect the IGF-1 system. In a variety of vertebrates EDCs have been shown to alter reproduction. Much research has focused on the interaction of EDCs with steroid hormones and their receptors (Rooney and Guillette, 2000). Unfortunately, the effect of EDCs on the IGF-1 system has received surprisingly little scientific scrutiny (Backlin and Bergman. 1995; Backlin, et al., 1998). Thus, another goal of my study was to determine whether nitrate and nitrite (known to induce developmental abnormalities in amphibians and reproductive abnormalities in other vertebrates) can alter concentrations of IGF-1 and steroid hormones and alter growth of reproductive tissues in amphibians.

The effect of nitrate on synthesis of IGF-1 and steroids remains an important topic for investigation. Growing evidence indicates that nitrate exposure stimulates NO synthesis in body tissues. Furthermore, increased NO expression in gonadal tissues affects steroid and IGF-1 expression. Thus, nitrate exposure might influence IGF-1 synthesis, similar to steroids, through an NO-dependent or independent pathway.

Finally, my study examined adult anurans for seasonal changes in IGF-1 and steroid concentrations, and in reproductive tissues. Seasonal patterns of change in plasma IGF-1 and steroid hormone concentrations, and in growth of reproductive tissues, are reported for alligators and turtles (Crain et al., 1995; Guillette et al., 1996). In anurans, seasonal changes in plasma IGF-1 have been reported for the Woodhouse toad, *Bufo woodhousei* (Pancak-Roessler and Lee, 1990). Thus, I expected that plasma IGF-1 and steroid concentrations, and growth of reproductive

tissues, would exhibit a seasonal pattern of change in response to endogenous stimulation and environmental cues.

In addition to addressing the goals mentioned above, findings from my study also have more general applications for studies of amphibian physiology, evolution, and conservation.

Physiology and Evolution

Physiological regulation of the IGF-1 system has been examined in mammals and reptiles. The IGF-1 has been shown to regulate gonadal steroid synthesis, to stimulate oviductal growth, and to exhibit seasonal cyclicity in mammals and reptiles. Recent research on reptiles and mammals demonstrates that IGF-1 potentiates E_2-induced growth of reproductive tissues like the oviduct. Even in the absence of endogenous E_2, IGF-1 stimulates significant oviduct growth. Thus, the role of growth factors in reptilian and mammalian reproduction is more important than previously recognized. Additionally, seasonal cycles of increased plasma steroid concentrations and increased reproductive tissue growth overlap with increases in plasma IGF-1 in reptiles and mammals (Crain et al., 1995; Guillette et al., 1996; Webster et al., 2001). These findings indicate that IGF-1 is associated with reproductive activity, and is responsive to changes in reproductive parameters and environmental cues.

In amphibians, the presence of IGF-1 has been documented; but the physiological processes that regulate this system remain largely under-investigated. If the amphibian oviduct responds to IGF (similar to mammals and reptiles), then IGF-1 regulation of reproductive tissues represents an early evolutionary phenomenon. However, if the amphibian oviduct is unresponsive to IGF-1 stimulation, then IGF-induced oviduct growth might represent a relatively recent development in reptiles and mammals. Seasonal changes in plasma IGF-1 concentration have been described for *B. woodhousei*, but it remains unknown if changes in IGF-1 parallel reproductive parameters in this or other amphibian species (Pankcak-Roessler and Lee, 1990). My study provides the first description of how endogenous steroids, environmental factors, and reproductive cyclicity influence the IGF-1 system in amphibians.

Conservation

Amphibian populations in some agricultural areas are declining, and frogs have been found with dramatic deformities. The factors responsible for these declines and deformities are hard to identify, but might include runoff of nitrogenous fertilizers from agricultural land into watersheds where amphibians live and reproduce. Mammals drinking nitrate- and nitrite-contaminated water exhibit decreased gonadal steroid synthesis after only relatively brief exposure periods. Despite findings of abnormal growth and metamorphosis in tadpoles exposed to nitrate, no study has investigated whether nitrate alters endocrine function in juvenile or adult amphibians. Furthermore, most studies focus on the effects of lethal rather than sublethal concentration of nitrate on amphibians. My study examined the effects of sublethal concentrations of nitrate and nitrite on plasma steroids, gonadal steroid synthesis, and growth of reproductive tissues in amphibians. If nitrate or nitrite exposure alters endocrine function, specifically reproductive steroids, then these contaminants should be considered as an important factor to consider in amphibian reproduction and population declines.

Table 1-1. The molecular formula weight (MFW) of sodium nitrate ($NaNO_3$) and sodium nitrite ($NaNO_2$), the MFW percent of sodium (Na), nitrate (NO_3), nitrite (NO_2), and nitrogen (N), and the equivalent concentrations of nitrate, nitrite, nitrate as nitrogen (NO_3-N), and nitrite as nitrogen (NO_2-N) as milligrams per liter (mg/L) and millimolar (mM) of solution.

	Percent sodium (Na)	Percent nitrate (NO_3)	Percent nitrogen (N)	$NaNO_3$ (mg/L)	Nitrate NO_3 (mg/L)	Nitrate as nitrogen (NO_3-N) (mg/L)	Nitrate NO_3 (mM)	Nitrate as nitrogen (NO_3-N) (mM)
Sodium nitrate ($NaNO_3$) FW 84.99 g	27.0 %	73.0 %	16.5 %	0	0.00	0.00	0.00	0.00
				1	0.73	0.17	0.009	0.002
				10	7.30	1.65	0.01	0.003
				40	29.20	6.60	0.34	0.08
				100	73.00	16.50	0.86	0.19
				150	109.50	24.75	1.29	0.29
				200	146.00	33.00	1.72	0.39
				300	219.00	49.50	2.58	0.58

	Percent sodium (Na)	Percent nitrite (NO_2)	Percent nitrogen (N)	$NaNO_2$ (mg/L)	Nitrite NO_2 (mg/L)	Nitrite as nitrogen ($N0_2$-N) (mg/L)	Nitrite NO_2 (mM)	Nitrite as nitrogen ($N0_2$-N) (mM)
Sodium nitrite ($NaNO_2$) FW 68.99 g	33.3 %	66.7 %	20.3 %	0	0.00	0.00	0.00	0.00
				1	0.67	0.20	0.01	0.003
				10	6.67	2.03	0.10	0.03
				40	26.68	8.12	2.90	0.12
				100	66.70	20.30	0.39	0.29
				150	100.05	30.45	0.97	0.44
				200	133.40	40.60	1.45	0.59
				300	200.10	60.90	2.90	0.88

Table 1-2. A comparison among amphibians of the effects of nitrate and nitrite

Species	Stage	Treatment	End Point	Reference
Ambystoma gracile	Larvae	0.78-25 mg/L nitrate 4 mg/L nitrite	Decreased feeding & activity, bent tails, edema LC_{50} < 15 days	Marco and Blaustein, 1999
Bufo americanus	Tadpoles	Acute:13.6-39.3 mg/L nitrate Chronic: 2-10 mg/L nitrate	LC_{50} 96 h, decreased swimming and feeding Bent tail, edema, head and digestive deformities	Hecnar, 1995
B. boreas	Tadpoles	0.78-25 mg/L nitrate 4 mg/L nitrite	Decreased feeding & activity, bent tails, edema LC_{50} < 15 days	Marco and Blaustein, 1999
B. bufo	Tadpoles	385 mg/L	LC_{50} 96 h	Xu and Oldham, 1997
Hyla regilla	Tadpoles	0.78-25 mg/L nitrate 4 mg/L nitrite	Decreased feeding & activity, bent tails, edema LC_{50} < 15 days	Marco and Blaustein, 1999
Litoria caerulea	Tadpoles	9-22.6 mg/L nitrate	Decreased growth rates, behavior abnormalities, increased mortality	Baker and Waights, 1994
Pseudacris triseriata	Tadpoles	Acute: 13.6-39.3 mg/L nitrate Chronic: 2-10 mg/L nitrate	LC_{50} 96 h, decreased swimming and feeding, bent tail Edema, head and digestive deformities	Hecnar, 1995
Rana aurora	Tadpoles	0.78-25 mg/L nitrate 4 mg/L nitrite	Decreased feeding & activity, bent tails, edema LC_{50} < 15 days	Marco and Blaustein, 1999
R. cascadae	Tadpoles	3.5 mg/L nitrate	Decreased rates metamorphosis at earlier stage development	Marco and Blaustein, 1999
R. catesbeiana	Tadpoles	9-26 mg/L nitrate	Decreased white blood cells and hemoglobin	Dappen, 1983
R. pipiens	Tadpoles	9-26 mg/L nitrate	Decreased white blood cells and hemoglobin	Dappen, 1983
R. pipiens	Tadpoles	Acute: 13.6-39.3 mg/L nitrate Chronic - 2-10 mg/L nitrate	LC_{50} 96 h, decreased swimming and feeding, bent tail Edema, head and digestive deformities	Hecnar, 1995
R. clamitans	Tadpoles	Acute: 13.6-39.3 mg/L nitrate Chronic: 2-10 mg/L nitrate	LC_{50} 96 h, decreased swimming and feeding, bent tail Edema, head and digestive deformities	Hecnar, 1995
R. pretiosa	Tadpoles	0.78-25 mg/L nitrate 4 mg/L nitrite	decreased feeding & activity, bent tails, edema LC_{50} < 15 days	Marco and Blaustein, 1999
R. temporaria	Tadpoles	5 mg/L nitrate	Decreased growth rates and decreased size at metamorphosis	Johansson et al., 2001
R. temporaria	Adults	3.6-6.9 g/m^2 nitrate on substrate	Increased toxicity and mortality	Oldham et al., 1997

CHAPTER 2
THE EFFECTS OF EXPOSURE TO ENVIRONMENTALLY RELEVANT CONCENTRATIONS OF NITRATE (*IN VIVO*) ON PLASMA STEROIDS AND INSULIN-LIKE GROWTH FACTOR-1, ON OVARIAN STEROID SYNTHESIS, AND ON OVIDUCT GROWTH IN THE AFRICAN CLAWED FROG (*Xenopus laevis*)

Introduction

During the last few years there has been increased global concern over contamination of water by anthropogenic sources of nitrates. Nitrate is among the most stable, water-soluble ionic forms of nitrogen persistent in aquatic habitats. Nitrate contaminates watersheds in agricultural and urban environments, reaching harmful concentrations largely due to human activities. In agricultural areas, nitrate contaminates watersheds primarily through poorly regulated runoff of nitrogen-based fertilizers and animal wastes from farms. In urban areas, nitrate contaminates watersheds primarily through release of industrial and wastewater effluent from treatment plants, runoff of fertilizers applied to lawns and golf courses, and air pollution from the burning of fossil fuels (Pucket, 1995; Rouse et al., 1999). In temperate North America, concentrations of aquatic nitrate are highest between the fall and spring when reduced ion uptake by agricultural plants increases soil nitrate loads leaching from the ground (Hallberg, 1989; Nolen and Stoner, 1995; Nolen et al., 1995, 1997). Additionally, fertilizers applied in close proximity to watersheds, coupled with spring rainstorms, contributes to an overwhelming aquatic nitrate pulse that frequently exceeds 100 mg/L and overlaps the breeding season of many amphibians (Rouse et al., 1999). Many studies on the effects of nitrate on amphibians have addressed the effects of toxicological rather than sublethal doses on growth, skeletal, and tissue deformities in juvenile amphibians (Cooke, 1981; Baker and Waights, 1993; Hecnar, 1995; Watt and Oldham, 1995; Oldham et al., 1997; Xu and Oldham, 1997; March and Blaustein, 1998; Marco et al., 1999;

Johansson et al., 2001; Chapter 1, Table 1-2). Surprisingly, few studies have investigated effects of exposure to sublethal nitrate concentrations on adult, reproductive frogs.

There is mounting evidence that nitrate interferes with steroid-signaling pathways. Panesar and Chan (Panesar, 1999; Panesar and Chan, 2000) demonstrated that administration of nitrate and nitrite inhibits testosterone (T) synthesis (*in vitro* and *in vivo*) in rodents. Once nitrate enters the body, through consumption or absorption across skin surfaces, it can be converted into nitrite by endogenous microbial activity in the mouth or gastrointestinal tract (Fried, 1991; Doblander and Lackner, 1996). Nitrite can be converted into N-nitrosoamines, which are carcinogens in laboratory animals and in humans (National Academy of Sciences, 1981; Tricker and Preussmann. 1991; US EPA, 1995). One proposed mechanism for altered steroid expression by nitrates involves enzyme-dependent synthesis of nitric oxide (NO) (Panesar and Chan, 2000). The NO is synthesized (*in vivo*) from an L-arginine precursor by nitric oxide synthase (NOS) enzymes (Kleinert et al., 1995; Mayer and Hemmenns, 1997). In addition to NOS-dependent NO formation, non-enzymatic synthesis of NO can also occur through acidic reduction of nitrite (Iizuka et al., 1999; Zweier et al., 1995, 1999; Modin et al., 2001). Cosby et al. (2003) reported that hemoglobin functions as a nitrite reductase contributing to enzyme-independent NO synthesis. Regardless of the mechanisms by which it is produced, NO is thought to regulate many physiological processes. Zweier et al. (1999) reported that enzyme-independent NO formation is associated with cellular damage and loss of organ function. Panesar and Chan (2000) proposed that, in steroidogenic tissues, NO binds to the heme groups inherent to mitochondrial cytochrome P450 enzymes, such as those involved in side-chain cleavage ($P450_{scc}$): the rate-limiting step in steroid synthesis. The NO can inhibit other P450 enzymes, such as 3β-dehydroxysteroid dehydrogenase (3β-HSD) involved in androgen synthesis; and P450 aromatase (Snyder et al., 1996) involved in aromatization of androgens to estrogens. Collectively, these P450 enzymes are

necessary for conversion of free cholesterol into progesterone (P_4): the steroid precursor for T and 17β-estradiol (E_2).

Various isoforms of NOS are found within the ovary and other steroidogenic tissues in vertebrates (Szabo and Thiemermann. 1995; Van Voorhis et al., 1995; Srivastava et al., 1997). Disruption of these enzymes by NO might inhibit P_4 synthesis, which would decrease or prevent downstream T synthesis. Inhibition of gonadal T synthesis likely reduces the T available for aromatase conversion to E_2 and would contribute to decreased overall gonadal E_2 synthesis. This speculation is supported by studies in mammals demonstrating that increased NOS activity and NO concentrations are associated with decreased ovarian E_2 synthesis (VanVoorhis et al., 1994, 1995; Jablonka-Shariff and Olsen, 1997; Srivastava et al., 1997; Dees et al., 2000).

Relatively few studies have reported the impact of nitrate on steroidogenesis, but no study has investigated the effect of nitrate exposure on of insulin-like growth factor-1 (IGF-1) in vertebrates. Insulin-like growth factor-1 is a potent growth-stimulating hormone that regulates bone and skeletal muscle growth, limb bud emergence, reproductive and somatic tissue growth, steroidogenesis, and other physiological functions (Daughaday and Rotwein, 1989; Erickson et al., 1989; Adashi, 1993; Hiney et al., 1996; Olsen et al., 1996; Dees et al., 1998; Kaliman et al., 1999; Allen et al., 2001). Thus, IGF-1 is a relevant hormone to examine in the cases of amphibian skeletal deformities, sex ratio reversal, and reproductive abnormalities. Abnormal expression of IGF-1 is associated with altered growth and function of reproductive tissues in vertebrates. Increased concentrations of plasma IGF-1 in humans is positively correlated with cancer of the endometrium, breast, prostate, skin, pancreas, lung, and colon (Cohen et al., 1991; Lippman, 1993; Papa et al., 1993; LeRoith et al., 1993, 1995; Werner and LeRoith, 1996; Cascinu et al., 1997; Mantzoros et al., 1997; Stoll, 1997). Despite these reports, IGF-1 can have beneficial effects on tissue growth and function. For example, IGF-1 also mediates growth of E_2- sensitive reproductive tissues. In addition to this, IGF-1 regulates gonadal steroid expression. Intraovarian IGF-1 expression counteracts NO-induced steroid inhibition by increasing aromatase activity and

stimulating E_2 synthesis (Daughaday and Rotwein, 1989; Erickson et al., 1989; Adashi, 1993; Hiney et al., 1996; Olsen et al., 1996; Dees et al., 1998). Furthermore, evidence indicates that NO stimulates ovarian IGF-1 expression (Dees et al., 1998). Thus, NO interacts with the IGF-1 system and influences expression of steroids and also their actions in reproductive tissues.

Based on the aforementioned studies, I hypothesized that nitrate alters concentrations of steroids and IGF-1, and alters oviduct growth in a model frog species, *Xenopus laevis*. My study tested this hypothesis using environmentally relevant concentrations of nitrate.

Materials and Methods

Animals and Samples

Adult female *X. laevis* were purchased from Xenopus Express (Plant City, Florida). This species is entirely aquatic, and thus would remain in constant exposure to administered treatments. Frogs were maintained under a 12-h light/dark cycle in 38 L tanks with 19 L of static-flow, dechlorinated water at 23°C (pH 7.0 – 7.4), with ammonia and nitrite content below 1.0 mg/L as confirmed by daily water measurements. Animals were fed spirulina pellets (Aquatic Ecosystems, Orlando, FL) every other day for the duration of the experiment. All procedures were performed with approval of the University of Florida Institute of Animal Care and Use Committee (IACUC Permit #Z023). Pregnant mare serum gonadotropin (PMSG) and human chorionic gonadotropin (hCG) were obtained from Sigma-Aldrich (St. Louis, MO), and sodium nitrate (99% purity) was obtained from Fisher Scientific (Orlando, FL).

Nitrate Study Design

Treatment groups were divided into control (0 mg/L), 150 mg/L, and 300 mg/L sodium nitrate; respectively equivalent to 0, 24.75, and 49.50 mg/L nitrate-as-nitrogen (NO_3-N). Nitrate as nitrogen represents the concentration of nitrogen present in a given concentration of sodium nitrate administered (Chapter 1, Table 1-1). For the remainder of this chapter, nitrate will refer to NO_3-N.

The frogs were randomly assigned to each of 3 replicate tanks per treatment for a total sample size of 12 frogs per treatment. No significant differences in mass were detected (ANOVA; $P > 0.05$) or snout-vent-length (SVL; ANOVA; $P > 0.05$) among frogs in each treatment group. After a 1-week acclimation period, frogs were injected into the dorsal lymph sac with 50 IU of PMSG, followed 3 days later by an injection of 750 IU hCG. These treatments stimulated ovulation and formation of new ovarian follicles within 6 weeks (Dumont, 1971; Fortune and Tsang, 1981; Fortune, 1983). This procedure synchronized the size and maturation of new follicles before nitrate exposure, and minimized possible variation in gonadal steroid synthesis among frogs in response to treatment.

After 6-weeks, frogs were exposed to nitrate applied to tank water for 7 consecutive days. Every 24 h, water was changed, and fresh water with nitrate was added. After 7 days, the frogs were anesthetized with MS-222 (1.5% 3-aminobenzoic acid ethyl-ether, Aquatic Ecosystems, Orlando, FL). Blood was collected by cardiac puncture using heparinized syringes, placed into heparin vacutainer tubes, and centrifuged (2500×G) for 15 min; and plasma was stored at -70ºC for E_2, T, and IGF-1 radioimmunoassay (RIA) analysis. The ovaries were removed and weighed, and follicles were dissected for a culture study (*ex vivo*). Follicles of specific maturation stages were chosen: stage 4 follicles synthesize E_2, and stage 5 and 6 follicles synthesize T (Fortune and Tsang, 1981; Fortune, 1983;). From each frog, 33 follicles, each of stages 4, 5, and 6 were incubated in 35×10 mm sterile culture dishes, in duplicate, at 23ºC with 2 mL of sterile, phenol-free culture media (1L M199 HBSS, 3.4 mL 200 mM L-glutamine, 5.96 g/L HEPES, 0.35 g/L sodium bicarbonate, 8.0 mL 0.1 mM IBMX, pH 6.9; Sigma-Aldrich, St. Louis, MO) for both 5 and 10 h. Follicles were incubated at the same temperature Incubation temperature was selected based on the water temperature maintained in the tanks holding *X. laevis*. After incubation, culture media was decanted, flash-frozen, and stored at -70ºC for E_2 and T RIA. The diameter of the remaining, uncultured follicles was measured with a dissecting microscope and an ocular

micrometer. For each follicle stage, 5 follicles (un-cultured) were measured in each frog from 0 mg/L (control, N = 8), 24.75 mg/L nitrate (N = 10), and 49.50 mg/L nitrate (N = 8) treatment groups. Sample sizes of frogs were uneven among treatment groups for follicle measurements because for some frogs, all of the follicles were incubated in the culture study. Ovary, liver, and oviduct weights were recorded to compare post-treatment tissue weights among groups.

Steroid Radioimmunoassay (RIA) Procedures

RIAs were performed for E_2 and T (Guillette et al., 1994; Guillette et al., 1996) on culture media and on plasma samples using validated procedures. Duplicate media samples or plasma (50 µL for E_2 T) were extracted twice with ethyl-ether, air-dried, and reconstituted in borate buffer (0.05 M; pH 8.0). Antibody (Endocrine Sciences) was added at a final concentration of 1:55,000 for E_2 and of 1:25,000 for T. Radiolabeled steroid ([2,4,6,7,16,17-^{3}H] estradiol at 1 mCi/mL; [1,2,6,7-^{3}H] and testosterone at 1 mCi/mL; Amersham Int., Arlington Heights, IL) was added at 12,000 cpm per 100 µL for a final assay volume of 500 µL. Interassay variance tubes were prepared from two separate pools of media and of plasma for E_2 and T. Standards for E_2 and T were prepared in duplicate at 0, 1.56, 3.13, 6.25, 12.5, 25, 50, 100, 200, 400, and 800 pg/tube. Assay tubes were vortexed and incubated overnight at 4°C.

Bound-free separation was performed using a mixture of 5.0% charcoal to 0.5% dextran, pulse-vortexing, and centrifuging tubes (1500g, 4°C, 30 min). Supernatant was added to 5 mL of scintillation cocktail, and counted. Media intraassay and interassay variance averaged 2.50% and 3.70% for E_2, and 4.20% and 8.38% for T, respectively. Plasma intraassay variance for E_2 and T averaged 4.20% and 4.60%, respectively. Plasma E_2 and T samples were run in a single assay and interassay variances are not reported.

Validation of the steroid assays included media and plasma dilutions (50, 100, and 200 µL for E_2 and 20, 50 and 100 µL for T) compared with E_2 and T standards.

Insulin-Like Growth Factor-1 RIA Procedures

The IGF-1 RIA was performed as described by Crain et al. (1995). The National Hormone and Pituitary Program (Torrance, CA 90509) supplied human recombinant IGF-1 standard (9.76 to 2500 pg/tube) and human IGF-1 antisera (Lot # AFP4892898, 1:400,000 final dilution). The antiserum had less than 1.0% cross-reactivity with human IGF-II. Iodinated IGF-1 label (IGF-1^{I125} sp act 2000 Ci/mmol; 16,000 cpm/tube) and Amerlex-M donkey anti-rabbit secondary antibody (RPN510) were obtained from Amersham International (Arlington Heights, IL).

For each treatment group, plasma was pooled (8, 16, 24, and 36 μL aliquots in borate buffer) for validation using plasma dilutions (equivalent to 1.9, 3.9, 5.7, and 8.3 μL plasma) that were compared with IGF-1 standard. Plasma validation and experimental samples (20 μL) were acid-ethanol extracted and IGF-1 RIA performed (Crain et al., 1995). Validation samples were run in one assay with intraassay variance averaging 3.10%.

Biochemical RIA Validations

Plasma dilutions and internal standards were parallel to E_2 standards (ANCOVA; F = 0.48; $P = 0.52$ and F = 0.35; $P = 57$, Fig. 2-1A) and recovery of E_2 after extraction was 81.0%. Plasma dilutions and internal standards were parallel to T standards exhibited parallel displacement (ANCOVA; F = 0.12, P = 0.33 and F = 1.18, $P = 0.31$, Fig. 2-1B) and recovery of T after extraction was 93.8%. Plasma dilutions and IGF-1 standards exhibited parallel displacement curves (ANCOVA; F = 0.08; $P = 0.79$, Fig. 2-1C) and recovery of IGF-1 after extraction was 78.0%.

Media dilutions and E_2 standards gave parallel displacement curves (ANCOVA; F = 1.05; $P = 0.37$, Fig. 2-2A). Recovery of E_2 after media extraction was 91.5% and all sample values were corrected for loss using this value. Media dilutions and T standards gave parallel displacement curves (ANCOVA; F = 0.60; $P = 0.48$, Fig. 2-2B). Recovery of T after media

extraction averaged 98.9% and all sample values were corrected for this loss. For subsequent steroid and IGF-1 analyses, all sample values were corrected for respective losses.

Statistics

Ovary, oviduct, and liver wet mass were compared among treatment groups with body mass as a covariate using ANCOVA, followed by LSD post-hoc contrasts. Concentrations of E_2, T, and IGF-1 were estimated from raw data using Microplate Manager software (Microplate Manager III, BioRad Laboratories, Inc., Hercules, CA, 1988). Statistical analyses were performed using SPSS software (v. 10, SPSS Inc., Chicago, IL, 1999) with $\alpha = 0.05$. ANCOVA was used to validate plasma and media samples and to determine if plasma IGF-1 concentrations were correlated to body mass. Concentrations of E_2, T, and IGF-1 among replicate tanks within each treatment group were compared using one-way ANOVA. Where no significant difference existed among replicate tanks within treatment groups, mean E_2, T, and IGF-1 concentrations were compared among treatment groups with one-way ANOVA. Ovarian follicle diameters were compared, separately according to stage, among treatment groups with one-way ANOVA. Following one-way ANOVA analyses Scheffe post-hoc contrasts were used. Tamhane post-hoc contrasts were used where variances were unequal among groups for plasma IGF-1 concentrations.

Results

Tissue Weights

Tissue weights were not different among treatment groups for ovary (ANCOVA; $F = 0.57$, $P = 0.57$), oviduct (ANCOVA; $F = 0.28$, $P = 0.76$), and liver (ANCOVA; $F = 1.13$, $P = 0.34$).

Follicle Diameters

Diameter of stage 4 follicles was larger (ANOVA; $P = 0.01$, Fig. 2-3A) in frogs exposed to 24.75 mg/L and 49.50 mg/L nitrate relative to frogs exposed to 0 mg/L. Mean diameter was smaller in stage 5 (ANOVA; $P = 0.04$, Fig. 2-3B) and stage 6 follicles (ANOVA; $P = 0.005$, Fig.

2-3C) in frogs exposed to 49.50 mg/L nitrate compared to frogs exposed to 24.75 mg/L nitrate and 0 mg/L.

Plasma Steroid Concentrations

Analyses revealed no significant difference ($P > 0.05$) in E_2 or T concentrations among replicate tanks; thus data from frogs in replicate tanks was combined per treatment group. Plasma E_2 was not significantly different among treatment groups (ANOVA; $P = 0.08$). Plasma T did not differ among treatment groups (ANOVA; $P = 0.70$).

Plasma IGF-1 Concentrations

Analyses revealed no significant difference in IGF-1 concentrations among replicate tanks; thus data from frogs in replicate tanks was combined per treatment group. Plasma IGF-1 concentrations were significantly higher in frogs exposed to 24.75 mg/L and 49.50 mg/L nitrate relative to the control frogs (ANOVA; $P = 0.007$, Fig. 2-4). Plasma IGF-1 was not significantly correlated to body mass (ANOVA; $R^2 = 0.12$, $P > 0.05$).

Ovarian Follicle Steroid Concentrations (*Ex Vivo*)

Statistical analyses revealed no significant difference in mean E_2 or T ($P > 0.05$) concentrations among replicates for each treatment group; thus, data from frogs in replicate tanks was combined per treatment group. After 5 h, media E_2 concentrations were significantly lower for ovarian follicles of frogs exposed to 49.50 mg/L nitrate compared to the other treatment groups (ANOVA; $P < 0.001$, Fig. 2-5A). However, after 10 h, media E_2 concentrations were significantly lower for ovarian follicles of frogs exposed to both the 24.75 mg/L and 49.50 mg/L nitrate relative to the controls (ANOVA; $P < 0.001$, Fig. 2-5B).

After 5 h, media T concentrations were similar among treatment groups (ANOVA; $P > 0.05$, Fig. 2-6A). However, after 10 h, media T concentrations were significantly lower for ovarian follicles from frogs exposed to 24.75 mg/L and 49.50 mg/L nitrate relative to the control group (ANOVA; $P < 0.001$, Fig. 2-6B).

Discussion

This study has shown that exposure of *X. laevis* to sublethal doses of aquatic nitrates at environmentally relevant concentrations (24.75 mg/L and 49.50 mg/L) is associated with endocrine disruption of E_2, T, and IGF-1. This study raises new and troubling questions regarding the effects of nitrates on endocrine function. No other study has examined the effects of exposure to sublethal concentrations of nitrate on adult anurans, despite reports of altered growth, behavior, and mobility in tadpoles at similarly low (1 - 40 mg/L) nitrate concentrations (Baker and Waights, 1994; Hecnar, 1995; Xu and Oldham, 1997; Marco and Blaustein, 1999 Johansson et al., 2001.

Over the past 30 years amphibian populations have declined in various regions of the world (Wake, 1991; McCoy, 1994), especially in agricultural landscapes (Dappen, 1983; Berger, 1989; de Solla et al., 2002). Alteration of aquatic habitats is considered a primary contributor to these declines (Blaustein and Wake, 1990; Carey and Bryant, 1995). Altered endocrine function in frogs has been associated with exposure to sublethal concentrations of various contaminants (Mohanty-Hejmadi and Dutta, 1981; Carey and Bryant, 1995; Reeder et al., 1998; Kloas et al., 1999; Hayes et al., 2002). In agricultural and urban areas, contamination of aquatic habitats by anthropogenic sources of nitrate poses a serious threat to wildlife and humans. Approximately 72 million tons of nitrogen-based fertilizers are used worldwide and, combined with release of industrial nitrogenous wastes, are likely responsible for increased nitrate contamination reported in surface waters, aquifers, and drinking water (Rouse et al., 1999). Most studies examining the effects of sublethal nitrate concentrations on frogs have focused on juvenile stages from egg through metamorphosing tadpole. There is an absence of research examining the effects of sublethal nitrate concentrations on the endocrine profile of adult, reproductive frogs.

Panesar and Chan (2000) reported inhibition of T synthesis (*in vitro*) in rodents after exposure to nitrate. Within body tissues, various isoforms of NOS enzymes are capable of converting nitrates into NO (VanVoorhis et al., 1994, 1995; Srivastava et al., 1997; Olsen et al., 1996; Jablonka-Shariff and Olson, 1997). In addition, acidic reduction and hemoglobin have been

shown to mediate non-enzymatic NO formation from nitrite (*in vivo*) (Zweier et al., 1995, 1999; Modin et al., 2001; Cosby et al. 2003). Many studies have shown that NO inhibits E_2 and T synthesis in rodents, humans, and cows (VanVoorhis et al., 1994; Wang and Marsden, 1995; Basini et al., 1998; Omura, 1999). Panesar and Chan (2000) proposed a mechanism (based on a synthesis of their work and that of other researchers) involving formation of NO. Nitrate and nitrite can be converted to NO within steroidogenic cells, and the NO inhibits steroidogenic P450 enzymes necessary for conversion of free cholesterol to steroid precursors. In addition to inhibiting P450 enzymes, NO has also been shown to inhibit steroid-acute regulatory protein (StAR) protein expression. During steroidogenesis, StAR protein is essential for transporting free cholesterol to the inner mitochondrial membrane (Wang and Marsden, 1995). I propose a similar nitrate-associated steroid inhibition, possibly involving NO formation, occurred within ovarian follicles of *X. laevis*. This steroid inhibition also and includes downstream inhibition of E_2 synthesis and stimulation of IGF-1.

In *X. laevis* exposed to nitrate ovarian steroid synthesis was inhibited (*ex vivo*) while plasma steroid concentrations (*in vivo*) were unaffected. These findings indicate that different mechanisms were involved in regulating *ex vivo* versus *in vivo* steroids in nitrate-exposed frogs. It is possible plasma steroid concentrations were unchanged due to compensatory responses of the hypothalamic-pituitary-gonadal (HPG) axis (Chapter 1, Fig. 1-1). Inhibition of steroid synthesis at the gonad level might have signaled a compensatory hypothalamic release of gonadotropin-releasing hormone (GnRH) causing pituitary release of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) into the blood. Increased plasma LH/FSH concentrations would stimulate ovarian synthesis of T and E_2, which could have contributed to normal circulating plasma steroid concentrations. In this study, ovarian *ex vivo* follicle steroid synthesis was recorded without measuring corresponding plasma gonadotropins. It is unknown if plasma steroid concentrations in nitrate-exposed frogs were maintained at levels similar to control frogs by compensation by the HPG axis. It is unlikely that compensatory responses of the HPG axis to

stimulate steroidogenesis by the gonads would influence plasma steroid concentrations because ovarian steroid synthesis was shown to be inhibited in nitrate-exposed frogs. Thus, gonadotropins would not be effective in stimulating steroid synthesis in nitrate-exposed frogs when steroidogenesis is inhibited at the level of the gonad. Therefore, another explanation must be considered.

The liver is the main organ for degradation of nitrate, and degradation of nitrate can elevate hepatic NO concentrations. Continuous administration of nitrate has been shown to increase hepatic NO synthesis and inhibit hepatic P450 enzymes activity (Minamiyama et al., 2004). Hepatic P450 enzymes are necessary for metabolism and excretion of circulating steroids. Thus, hepatic nitrate degradation can lead to NO formation and inhibition of hepatic P450 steroid metabolic enzymes. Reduced hepatic steroid metabolism could cause stasis or even augmentation of circulating steroid concentrations.

I propose a mechanism for the increase in plasma IGF-1 concentrations (*in vivo*) observed in nitrate-exposed *X. laevis*. Nitrate, once consumed or absorbed across skin surfaces, can be converted by microbial activity in the mouth and gastrointestinal tract to nitrite. Nitrite has been shown to stimulate hypothalamic NO formation and increase hypothalamic secretion of growth hormone-releasing hormone GHRH and pituitary release of growth hormone (GH) (de Caceres et al., 2003). Thus, the hypothalamic-pituitary-hepatic (HPH) axis regulates circulating IGF-1 concentrations, and this axis is influenced by nitrite and NO exposure (Fig. 2-7). Further research will be necessary to confirm the validity of this proposed pathway.

In addition to the liver, the ovary also produces IGF-1, although in relatively smaller quantities (Adashi, 1993). Stimulation of the ovary by pituitary FSH results in decreased synthesis of IGF-1-binding proteins and increased intraovarian IGF-1 synthesis and availability. Intraovarian IGF-1 might have an autocrine and endocrine effect of ovarian steroid synthesis (Grimes et al., 1992; Adashi, 1993; Basini et al., 1998). Increased IGF-1 has been shown to increase intraovarian aromatase activity and E_2 synthesis (Erickson et al., 1989; Monnieaux and

Pisselet, 1992; Adashi, 1993; Samaras et al., 1994; Samaras et al., 1996). However, plasma IGF-1 concentrations increased and ovarian E_2 concentrations decreased in *X. laevis* upon nitrate exposure. Perhaps the *in vivo* nitrate exposure period of 7 days was too brief to observe a compensatory increase in ovarian steroid synthesis with IGF-1 stimulation.

Plasma IGF-1 binding proteins (IGF-BP) play an important role in regulating the availability of IGF-1 to and within tissues. In this study, IGF-BP in the plasma and the ovaries were not measured, so the availability of increased plasma IGF-1 in nitrate-exposed animals merits investigation. If plasma IGF-1 increased in conjunction with a decrease in tissue IGF1-BP, then there might be an increase in IGF-1 utilization and growth response by tissues. In this study, there was no difference detected in ovary, oviduct, or liver tissue mass among nitrate treatment groups. This might indicate either that the increased circulating IGF-1 was not stimulating a growth response in these tissues or that circulating IGF-1 was bound to IGF-BP and unavailable for tissue uptake. Although no difference in total ovary weights was detected among treatment groups, follicle diameter varied among groups. The diameter of E_2-producing follicles (stage 4) were larger in nitrate-exposed frogs compared to control, which might reflect a growth response to increased IGF-1 exposure or compensatory tissue growth in response to declining E_2 levels. The diameter of T-producing follicles (stage 5 and 6) was smaller in frogs exposed to 49.50 mg/L nitrate compared to follicles of frogs exposed to 24.75 mg/L and 0 mg/L. This could reflect either the absence of IGF-1 uptake by these follicles or an absence of a growth-response to IGF-1.

This study raises new and troubling questions regarding the effects of nitrates on endocrine function in vertebrates. Chemical alteration of aquatic habitats is considered a foremost contributor to the declines and deformities reported for amphibian populations (Carey and Bryant, 1995; Wake, 1998; Hayes et al., 2002). Amphibians exposed to various contaminants, even at sublethal concentrations, exhibit malformations, reproductive abnormalities, sex ratio reversal, male feminization, and altered endocrine function (Reeder et al., 1998; Kloas et al., 1999; Hayes et al., 2002). The nitrate-associated endocrine disruption in *X. laevis* might differ from other

anuran species due to the interspecific variation in physiological response to nitrates (Chapter 1, Table 1-2). Further research is necessary to determine whether nitrate alters steroid and IGF-1 hormones in other anuran species, and to describe the range of sublethal nitrate concentrations capable of endocrine disruption. The nitrate concentrations used in this study were relevant to environmental concentrations measured in North American ground and surface water (Rouse et al., 1999; Nolen and Stoner. 1995). However, it would be valuable to ascertain if even lower nitrate concentrations have a similar endocrine disrupting capacity in frogs.

More research is needed to elucidate the mechanism by which nitrate inhibits steroid synthesis and increases circulating IGF-1 concentrations in amphibians and in other animals. Thus far, most reports of steroid inhibition by nitrate have focused on steroid synthesis and regulation exclusively at the gonad level. It is important to consider both upstream and downstream steroid regulation. Upstream regulation would changes in hypothalamic and pituitary hormone secretions in response to *in vivo* nitrate exposure. The important hormones to examine include hypothalamic GnRH and GHRH, and pituitary LH, FSH, and GH. Pituitary LH and FSH function in stimulating gonadal steroid synthesis and GH stimulates hepatic IGF-1 synthesis. Downstream regulation would include hepatic degradation and clearance of circulating steroids, and secretion of IGF-1. In addition to these topics, it is important to determine whether amphibian gonadal tissue contains NOS enzymes capable of synthesizing NO. It has been already been established that the amphibian brain contains NOS capable of generating NO (McLean et al., 2001; Gonzalez et al., 2002; McLean and Bilar, 2002). Furthermore, it is necessary to understand how nitrate exposure of frogs regulates intracellular expression of NOS, NO, steroidogenic enzymes, and steroid regulatory proteins. Lastly, since nitrate exposure is associated with changes in circulating IGF-1 concentrations, in addition to steroid synthesis, is vital to understand how these hormones collectively influence the reproductive physiology of amphibians.

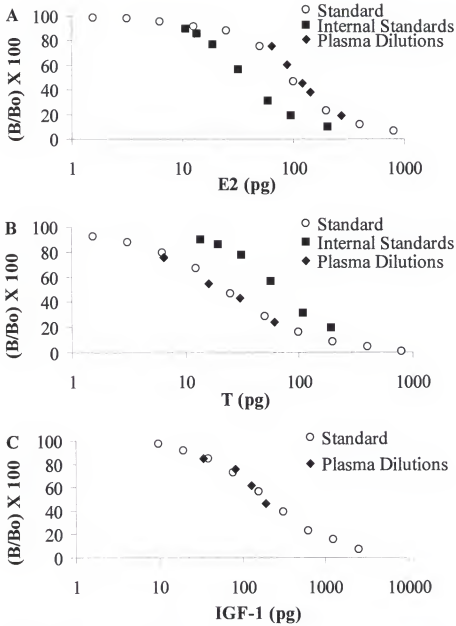

Figure 2-1. Biochemical validation of *Xenopus laevis* plasma. A. estradiol RIA internal standards (ANCOVA; F = 0.35; $P = 0.57$) and plasma dilutions (ANCOVA; F = 1.86; $P = 0.23$) were parallel to the standard curve. B. testosterone RIA. Internal standards (ANCOVA; F = 1.18; $P = 0.31$) and plasma dilutions (ANCOVA; F = 0.12; $P = 0.33$) were parallel to the standard curve. C. IGF-1 RIA. plasma dilutions (ANCOVA; F = 1.05; $P = 0.37$) were parallel to the standard curve.

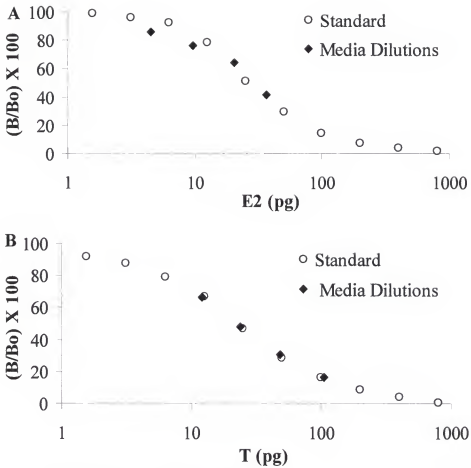

Figure 2-2. Biochemical validation of *Xenopus laevis* media. A. estradiol RIA media dilutions (ANCOVA; F = 0.08; $P = 0.79$) were parallel to the standard curve. B. testosterone RIA. Media dilutions (ANCOVA; F = 0.60; $P = 0.48$) were parallel to the standard curve.

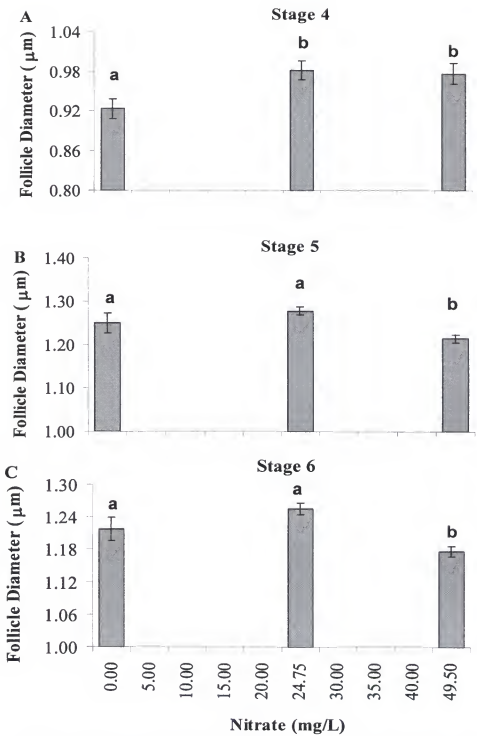

Figure 2-3. Diameter of ovarian follicles of stages 4, 5, and 6 in *Xenopus laevis* exposed *in vivo* for 7 days to 0, 24.75, and 49.50 mg/L nitrate. Data presented as means ± SEM. Different letters above bars indicate significant differences for: A. stage four (ANOVA; $P = 0.01$), B. stage five (ANOVA; $P = 0.040$, and C. stage six (ANOVA; $P = 0.005$).

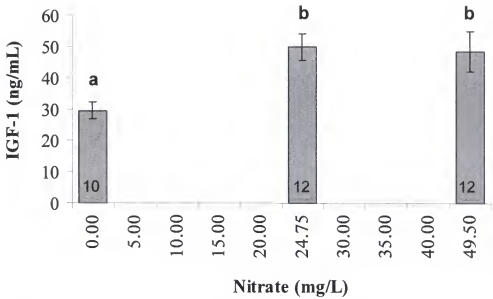

Figure 2-4. Plasma insulin-like-growth factor-1 (IGF-1) in *Xenopus laevis* after 7 days of *in vivo* exposure to 0, 24.75, and 49.50 mg/L nitrate. Data presented as means ± SEM. Numbers within bars indicate sample sizes and different letters above bars indicate significant differences (ANOVA; $P = 0.007$).

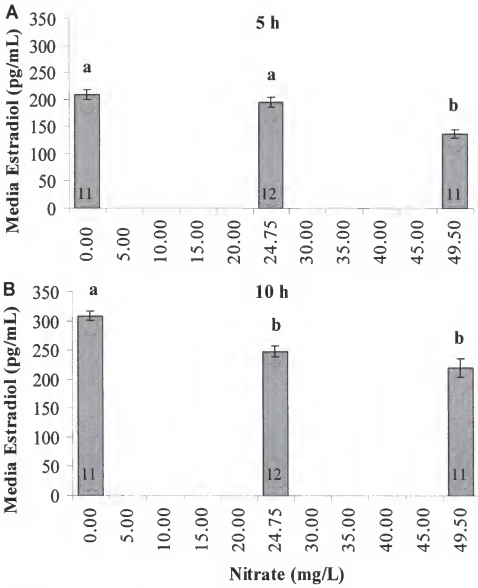

Figure 2-5. Media 17β-estradiol concentrations in culture media from incubated ovarian follicles of *Xenopus laevis* after 7 days *in vivo* exposure to 0, 24.75, and 49.50 mg/L nitrate. Data presented as means ± SEM for A. 5 h and B. 10 h of incubation. Numbers within bars indicate sample sizes and different letters above bars indicate significant differences for B. (ANOVA; $P < 0.001$).

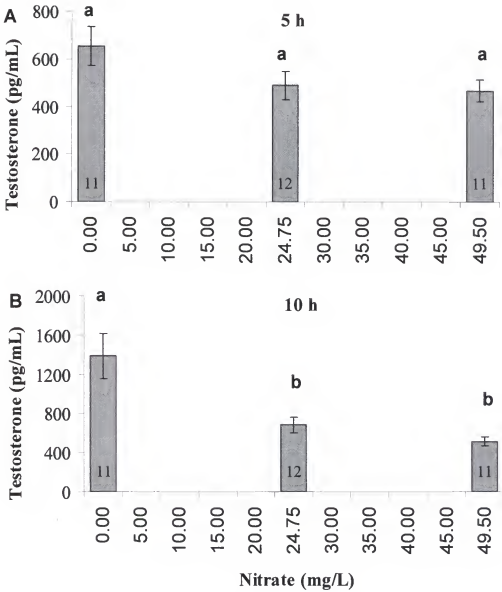

Figure 2-6. Media testosterone concentrations in culture media from incubated ovarian follicles of *Xenopus laevis* after 7 days *in vivo* exposure to 0, 24.75, and 49.50 mg/L nitrate. Data presented as means ± SEM. Numbers within bars indicate sample sizes and different letters above bars indicate significant differences for A. 5 h (ANOVA; $P = 0.007$) and B. 10 h (ANOVA; $P < 0.001$) of incubation.

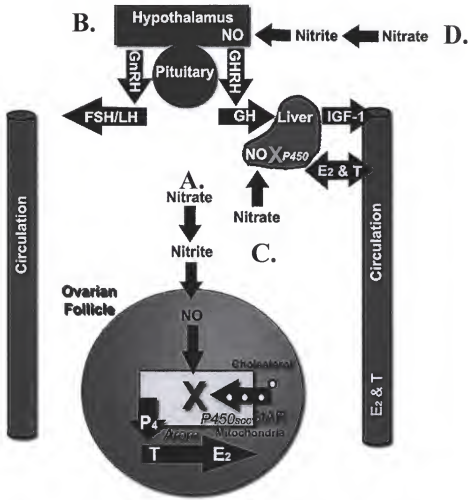

Figure 2-7. Diagram of mechanism for nitrate-associated inhibition of steroidogenesis and increased plasma IGF-1. Only ovarian testosterone, (T), estradiol 17β (E_2), and plasma T, E_2, and insulin-like growth factor-1 (IGF-1) were measured in *Xenopus laevis* exposed (*in vivo*) for 7 days to 0, 24.75, and 49.50 mg/L nitrate (NO_3-N). Other parameters are adapted from other studies (Licht 1984; Panesar and Chan, 2000; de Caceres et al., 2003; Minamiyama et al., 2004). A. Ovarian steroid synthesis is inhibited by nitric oxide (NO) formation from nitrate and nitrite. The NO inhibits cytochrome P450 steroidogenic enzymes. NO might also inhibit steroid-acute regulatory (StAR) protein which escorts free cholesterol into the mitochondria. Inhibition of these enzymes reduces progesterone (P_4) synthesis, and reduces T available for aromatization (Arom)to E_2. B. Decreased steroid synthesis could signal compensatory hypothalamic secretion of gonadotropin-releasing hormone (GnRH) pituitary secretion of luteinizing hormone (LH) and follicle stimulating hormone (FSH). C. Hepatic nitrate metabolism can cause NO inhibition of P450 enzymes involved in hepatic steroid metabolism and clearance resulting in augmented circulating steroid concentrations. D. Nitrate and nitrite could cause hypothalamic NO formation, which can stimulate secretion of growth hormone-releasing hormone (GHRH) and secretion of pituitary growth hormone (GH). The GH stimulates liver IGF-1 synthesis and secretion into the blood.

CHAPTER 3
SEASONAL CHANGES IN INSULIN-LIKE GROWTH FACTOR-1, STEROIDS, AND REPRODUCTIVE TISSUES IN PIG FROGS (*Rana grylio*)

Introduction

The sex steroids, 17β-estradiol (E_2) and testosterone (T), regulate virtually every facet of reproduction, and in ectotherms these hormones are responsive to changes in temperature, pH, and photoperiod among other environmental factors (Licht, 1970; Feder and Burggren, 1992; Norris, 1997; Kim et al., 1998).

Only one comprehensive profile of the pattern of seasonal changes in circulating steroid concentrations and changes in gonadal growth and maturation has been reported for a population of wild bullfrogs, *Rana catesbeiana* (Licht et al., 1983). Female *R. catesbeiana* exhibited a seasonal pattern of changes in plasma concentrations of gonadotropins and steroids, and in relative weights of reproductive tissues that indicate reproductive and non-reproductive periods. Reproductive period is here defined as physiological conditions that are optimal for reproduction. such as elevated plasma concentrations of the reproductive steroids E_2, T, and progesterone (P_4), and also by elevated weights of reproductive tissues such as the ovaries and oviducts. Reproductive condition of the frogs was also discerned by elevated plasma concentrations of the gonadotropins luteinizing hormone (LH), follicle stimulating hormone (FSH). In *R. catesbeiana*, E_2, T, and progesterone (P_4) concentrations were greatest in the reproductive period between May and July. The non-reproductive period of frogs is defined here as the physiological condition marked by decreased plasma concentrations of steroids and gonadotropins, and decreased weights of reproductive tissues. Licht et al. (1983) reported that plasma steroid concentrations and gonadal-somatic index (GSI) declined sharply after July and remained depressed between August and February, indicating the frogs were in non-reproductive condition. Similarly, plasma LH and

FSH concentrations declined precipitously by July of both years (Licht et al., 1983). Increased plasma steroid concentrations were likely stimulated by the elevated plasma gonadotropin observed. Elevated concentrations of LH stimulate gonadal steroidogenesis whereas elevated concentrations of FSH stimulate increased ovarian mass or gonadal somatic index (GSI) in females during the reproductive period (Licht, 1970, 1979; Norris, 1997). Plasma T concentrations in females greatly exceeded that of E_2 at all times, and plasma T concentrations were highly correlated with ovarian developmental stage. The relatively high T concentrations might serve as a circulating androgen pool for synthesis of E_2 by aromatase activity in peripheral tissues such as the brain, fat and skin, and even the oviduct (Follett and Redshaw, 1968). Plasma androgen pools also might serve functions unrelated to E_2 synthesis. For example, it has been reported that T synthesized by *Xenopus* ovaries might function to stimulate oocyte development directly through androgen receptors (Lutz et al., 2001).

In addition to steroid hormones, insulin-like growth factor-1 (IGF-1) regulates many aspects of reproduction including gonadal function and steroidogenesis (Adashi et al., 1991; Hammond et al., 1991). The presence of IGF-1 has been identified in representative animals from all vertebrate classes and includes humans, cows, rodents, birds, alligators, turtles, fish, and amphibians (Daughaday et al., 1985; Pancak-Roessler and Lee, 1990; Crain et al., 1995; Guillette et al., 1996; Le Roith et al., 2001a,b; Table 3-1). IGF-1 is a polypeptide hormone that stimulates cell growth in somatic and reproductive tissues and orchestrates many aspects of development, metabolism, and steroidogenesis (LeRoth et al., 2001a,b). In response to pituitary growth hormone (GH), the liver secretes IGF-1 into circulation complexed to IGF-1 binding proteins (IGF-1BPs). IGF-1 interacts with IGF-1 receptors located on tissues throughout body. Although initially described as an intermediate of GH action on skeletal muscle growth, more recently IGF-1 has been recognized as hormonal regulator of many GH-independent cellular processes (Butler and Le Roith, 2001; Le Roith et al., 2001a). Recent research has shown that IGF-1 synthesized within endometrial and ovarian tissue functions as a paracrine and autocrine hormone (Adashi,

1993). Studies in mammals demonstrate that increased intraovarian IGF-1 increases ovarian P_4 and E_2 synthesis, as well as aromatase, and steroid-acute regulatory protein (StAR) protein expression (Adashi et al., 1991; Adashi, 1993; Samaras et al., 1994, 1996; Devoto et al., 1999). Aromatase is a cytochrome P450 enzyme necessary for converting androgens into estrogens, and StAR proteins assist the entry of free cholesterol into the mitochondria to initiate steroid synthesis in steroidogenic tissues. Collectively, these findings demonstrate that IGF-1 is an important regulator of gonadal steroids. Other studies have shown that intraovarian IGF-1 regulates selection of dominant follicles for ovulation in mammals (Adashi et al., 1991; Giudice, 1999). These studies indicate that IGF-1 also plays a vital role in steroid synthesis, regulation, and gonadal function.

Although the IGF-1 system has been described in mammals, comparatively few studies have examined this system in non-mammalian vertebrates (Table 3-1). Oviparous vertebrates are intriguing models for examining the role of IGF-1 in reproduction because they lack a prolonged period of maternal and fetal chemical and nutritive interaction during embryonic development. Nutrients and growth promoting substances, like IGF-1, must be sequestered into eggs before oviposition and fertilization (Guillette et al., 1996). In a turtles, geckos, and alligators, the presence of plasma IGF-1 has been confirmed and demonstrated to play an important role in mediating reproduction (Daughaday et al., 1985; Cox and Guillette, 1995; Crain et al., 1995a,b; Guillette et al., 1996). Cox and Guillette (1995) demonstrated that ovariectomized (lacking endogenous E_2) geckos, exhibited an estrogen-like proliferation of oviductal tissue in response to treatment with IGF-1 implants. Additionally, plasma IGF-1 concentrations vary according to season and stages of reproductive maturation in female alligators and turtles (Crain et al., 1995; Guillette et al., 1996). These studies indicate IGF-1 plays a more important role in the growth of reproductive tissues than previously realized.

Unfortunately, the importance of IGF-1 in amphibian reproduction and growth remains largely under-investigated (Daughaday et al., 1985; Pancak-Roessler and Lee, 1990; Table 3-1).

Only one study reported seasonal changes of plasma IGF-1 concentrations in a wild population of *Bufo woodhousei* (Pancak-Roessler and Lee, 1990). Although this study was limited to a 10-month profile, it was evident that IGF-1 concentrations peaked during the reproductive period between May and June and decreased during the non-reproductive period between August and December (Pancak-Roessler and Lee, 1990).

No study has provided a simultaneous examination of seasonal changes in plasma steroid and IGF-1 concentrations, and in gonadal growth in a wild population of frogs. In order to understand the functional relationships among reproductive steroids, IGF-1, and reproductive tissues in frogs, it is essential to describe how these parameters fluctuate naturally under the influence of temporal and environmental factors. The objective of the following study was to document changes in concentrations of plasma IGF-1 and reproductive steroids (E_2 and T), and in gonadal tissues in conjunction with environmental factors for a population of wild female Pig frogs (*Rana grylio*) in a north-central Florida lake. *Rana grylio* were chosen for this study because they were abundant, they were relatively easy to acquire year-round, and they are the largest ranid frogs in Florida, which made them ideal for the tissue and blood collections required in this study. Additionally, *R. grylio* are closely related to bullfrogs (*R. catesbeiana*), a species for which documented seasonal profiles of E_2 and T served as a reference for this study (Castellani, 1958; Licht et al., 1983). Finally, the seasonal trends of gonadal maturation and breeding activity for *R. grylio* have been well-established (Ligas, 1960; Lamb, 1983). The seasonal pattern of changes in IGF-1 and sex steroids of wild-caught *R. grylio* established in this study serve as an ecologically relevant reference for comparison with findings presented in other chapters.

Materials and Methods

Water Parameters, Animal Captures and Sample Collections

From April of 2002 to July of 2003, 6 - 20 adult female *R. grylio* were collected during the fourth week of each month from Orange Lake (Lat. 29° 27°853'N, Long. 82° 11.380'W), in Alachua County, Florida (Fig 3-1). In October of 2002, frogs were not collected due to rain and

lightening storms encountered on the lake during 3 separate collection attempts. Animals were collected by hand from an airboat between 10 pm and 12 am. Captured frogs were transported, in covered buckets with a small amount of water, to the Dept. of Zoology where they were housed for less then 12 h in 38 L tanks with 19 L of dechlorinated water before examination.

Ligas (1960) reported that environmental factors such as rainfall, air temperature, and water temperature influence reproductive condition of *R. grylio* in the Everglades; therefore, these same parameters were measured at the collection site. Water temperature and pH were measured using a Myron L Ultrameter (model 6P, Carlsbad, CA 92009). Monthly rainfall and air temperature data from Orange Lake were recorded by Weather Station Number 02741536 and were kindly provided by David Clapp of the USGS and National Weather Service.

Additionally, water samples from the collection site were examined for nitrate and nitrite concentrations. Low precipitation combined with low water levels during the first 4 months of this study might have contributed to slightly eutrophic conditions within the collection site. Nitrate in known to interfere with gonadal steroidogenesis (Panesar and Chan, 2000) and with amphibian reproduction (Rouse et al., 1999). Thus, nitrate was an important parameter to measure when documenting plasma steroids of frogs collected at this site. Water nitrate and nitrite concentrations were measured, with the generous assistance of Thea Edwards, using an auto-analyzer (Technicon auto-analyzer II with colorimeter, Bran+Luebbe Inc., Chicago, (888)917-PUMP) equipped with a copper-cadmium reductor column. Methods for use are given in Bran+Luebbe method number US-158-71 C, which is equivalent to EPA method 353.2. The auto-analyzer has a detection limit of 0.43 µg/L with a detection range of 0-400 µg/L of nitrate as nitrogen. Samples are diluted in distilled water to fall within the detection range. Prior to analysis, samples are filtered through a 1 micron glass fiber filter, collected in new or acid-washed containers, and frozen (1 month) prior to measurement. Samples were quantified on a standard curve created with each batch of water samples.

Previous studies on wild-caught bullfrogs reported that increasing duration of captivity significantly decreased plasma hormones (Licht et al., 1983). Thus, a pilot study was performed to determine the influence of duration of captivity on plasma hormone concentrations in *R. grylio*. Blood samples were collected from frogs at 0, 6, 12, and 24 h post-capture. Blood samples were drawn from frogs immediately after capture and then at time intervals afterwards while being contained in covered buckets holding a small amount of lake water. No significant changes were detected in concentrations of plasma E_2, T, and IGF-1 over the 24 h period (Fig. 3-2). For consistency in all subsequent procedures, blood and tissue samples were collected from frogs within 12 h of capture. All animal procedures were performed in accordance with regulations specified by University of Florida, Institute of Animal Care and Use Committee (Permit #Z095) and a valid freshwater fishing license issued to T.R. Barbeau during the years of 2002 and 2003 as required by the State of Florida.

The frogs were anesthetized with MS-222 (1.5% 3-aminobenzoic acid ethyl-ether, Aquatic Ecosystems, Orlando, FL), snout-vent length (SVL) and body mass were recorded, and blood samples were obtained via cardiac puncture with heparinized syringe and needle. Blood samples were centrifuged and resultant plasma frozen (-70°C) for E_2, T, and IGF-1 radioimmunoassay (RIA) analyses. Frogs were then euthanized by dissection through the spinal cord followed by pithing.

The gonadal-fat bodies, liver, ovaries, and oviducts were removed from each frog and weighed. Fat bodies were examined because they are an important energy reservoir that can be metabolized to provide energy for growth of reproductive tissues before (and throughout) the reproductive period. The liver was examined because it is the primary site for synthesis of plasma IGF-1, vitellogenin, and other substances vital for reproduction in oviparous ectotherms (Crain et al.1995; Guillette et al., 1996). Ovarian maturation was categorized as either regressed (stage 1), yellow (stage 2), black (stage 3), or mature "black and white" (stage 4) based predominantly on the stages of follicular development described by Ligas (1960). Briefly, regressed ovaries were

small (< 0.75 mm diameter), yellow, and contained no visible follicles. Yellow ovaries were also small but contained yellow follicles up to 0.75 mm in diameter. Black ovaries were medium to large and contained mostly black follicles 1.0 - 1.25 mm in diameter. Black ovaries can mature within a relatively brief time to stage 4 ovaries. Lastly, mature ovaries were large, composed of highly polarized follicles 1.25 - 2.0 mm in diameter, and had a sharp delineation of light and dark colors indicating a vegetal and animal hemispheres. Mature ovaries contained oocytes ready for ovulation and fertilization (Fig. 3-3).

Small cross-sections of the ampulla region of the oviducts were fixed in 4% paraformaldehyde (4°C; 48 h) followed by rinse and storage in 75% ethanol for subsequent histological analyses. The ampulla region, or middle portion of the oviduct, was examined because it was the longest, most convoluted, and most visually distinct region (Wake and Dickie,1998). The ampulla region contains more glands and has a greater secretory activity than other oviductal regions. The oviduct samples were dehydrated in a graded series of ethanol changes, embedded in paraffin, serially cross-sectioned on a rotary microtome (7 μm), stained with modified Masson's staining procedure, and examined using light microscopy. To ascertain oviductal proliferation, an ocular micrometer was used to make 10 morphological measurements on 5 tissue sections, for a total of 50 measures per frog. The following oviductal parameters were measured: epithelial cell height, endometrial thickness, endometrial gland height, and endometrial gland width. Gland height and width measurements were used to calculate gland surface area (μm^2).

Steroid Radioimmunoassay (RIA) Biochemical Validation

Validation samples were obtained by creating plasma pools using aliquots from individual frogs collected. Two methods were used to validate the E_2 and T RIA: internal standards and plasma dilutions. One half of the plasma pool, for use with internal standards, was mixed with Norit charcoal (10 mL plasma to 1g charcoal ratio; 4°C; 24 h) to strip steroid

hormones from the plasma. The solution was then centrifuged (3000 rpm; 1200×G; 45 min) and the resultant supernatant decanted. Separate, duplicate aliquots of stripped plasma (25 μL) were added to tubes and spiked with 100 μL of assay buffer containing 1.56, 3.13, 6.25, 12.5, 25, 50, 100,200, 400, 800 pg E_2 or T hormone. These tubes were extracted twice with ethyl-ether, air-dried, and reconstituted in 100 μL borate buffer (100 μL; 0.05 M; pH 8.0).

For plasma dilutions, 6.25, 12.5, 25, 50, and 100 μL plasma was added to different tubes. Appropriate volumes of borate buffer were added to bring the final sample volume of each tube up to 200 μL. Samples were extracted twice with ethyl-ether, air-dried, and reconstituted with 100 μL borate buffer. Resultant samples for both internal standards and plasma dilutions were examined by the RIA procedure described below.

Plasma extraction efficiencies were determined by adding 100 μL tritiated E_2 and T (15,000 cpm) to 100 μL of pooled plasma samples, extracting twice with ethyl-ether, air-drying, and adding 500 μL scintillation fluid to tubes, and reading samples on a Beckman LS 5801 scintillation counter to determine the tritiated hormone remaining. The extraction efficiencies for E_2 and T samples were 90.0% and 91.4%, respectively. Supernatant (500 μL) was added to 5 mL of scintillation fluid, and counted on a Beckman scintillation counter. Plasma validation samples were run in one assay with intraassay variance for E_2 and T averaging 1.53% and 1.23%, respectively. Plasma interassay variance for E_2 and T averaged 6.99% and 3.27%, respectively.

Steroid RIA Procedures

RIAs were performed for E_2 and T on plasma samples collected before surgery and after treatments. For E_2 samples, 25 μL of plasma was used and for T samples, 6.25 μL of plasma was used. For T RIA, 50 μL of plasma samples were diluted with 200 μL of borate buffer, and 25 μL of this dilution (6.25 μL plasma equivalents) were used as samples in the RIA. These volumes were selected for analysis based on RIA volume determinations conducted on these samples previously. Briefly, duplicates of plasma samples were extracted twice with ethyl-ether, air-dried,

and reconstituted in borate buffer. To each tube, bovine serum albumin (Fraction V; Fisher Scientific) in 100 μL of borate buffer was added to reduce nonspecific binding at a final concentration of 0.15% for T and 0.19% for E_2. Antibody (Endocrine Sciences) was then added in 200 μL of borate buffer at a final concentration of 1:25,000 for T and 1:55,000 for E_2. Finally, radiolabeled steroid ([2,4,6,7,16,17-^{3}H] 17β-estradiol at 1 mCi/mL; [1,2,6,7-^{3}H] testosterone at 1 mCi/ml; Amersham Int., Arlington Heights, IL) was added at 12,000 cpm per 100 μL for a final assay volume of 500 μL. Interassay variance tubes were similarly prepared from 2 separate plasma pools for E_2 and T. Standards for both E_2 and T were prepared in duplicate at 0, 1.56, 3.13, 6.25, 12.5, 25, 50, 100, 200, 400, and 800 pg/tube. Assay tubes were vortexed for 1 min and incubated at 4°C overnight.

Bound-free separation was performed by adding 500 μL of a mixture of 5% charcoal to 0.5% dextran, pulse-vortexing, and centrifuging tubes (1500g, 4°C, 30 min). Supernatant (500 μL) was added to 3 mL of scintillation fluid, and counted on a Beckman scintillation counter. Plasma samples were run in 3 assays with intraassay variance for E_2 and T averaging 3.35% and 4.99%, respectively. Plasma interassay variance for E_2 and T averaged 3.97% and 6.99%, respectively.

Insulin-like Growth Factor-1 (IGF-1) RIA Biochemical Validation

Pooled plasma samples (200 μL) were extracted in polypropylene tubes with acid-ethanol (12.5% 2 N HCl, 87.5% ethanol; 400 μL) to dissociate IGF binding proteins from the IGF-1 molecules and to precipitate globular proteins as per Daughaday et al. (1980) and Crain et al. (1995). After 30 min incubation (23°C) and 10 min centrifugation (2500×G; 4°C), the supernatant was aliquoted to produce plasma equivalents of 12.5, 25, 50, 100, and 200 μL. Volume of the plasma dilutions was brought to 200 μL with acid-ethanol before air-drying. Plasma dilutions were compared with 0, 39, 156, 313, 625, 1000, 1250, 2500 pg of human recombinant IGF-1

standard (National Hormone and Pituitary Program, Torrance, CA 90509). Validation samples were examined by IGF RIA procedures as described for experimental sample analyses below.

Plasma extraction efficiencies were determined by adding 100 μL iodinated IGF-1 (15,000 cpm) to 100 μL of pooled plasma samples, extracting with acid-ethanol, air-drying, and reading samples on a Beckman 5500B gamma counter to determine the iodinated hormone remaining. The extraction efficiency of plasma was 78.0% and all sample concentrations were corrected for this loss. Validation of plasma dilutions was accomplished in one assay having an intraassay variance of 2.3%. Internal standards and plasma dilutions were parallel to the standard curve for E_2 (ANCOVA; $F = 0.24$, $P = 0.63$ and $F = 2.89$, $P = 0.15$, Fig. 3-4A), and T RIA (ANCOVA; $F = 0.001$, $P = 0.99$ and $F = 0.013$, $P = 0.92$, Fig. 3-4B).

IGF-1 RIA Procedures

IGF-1 RIA was performed as described by Crain et al. (1995) and Guillette et al. (1994). The National Hormone and Pituitary Program (Torrance, CA 90509) supplied human recombinant IGF-1 standard (9.76 to 2500 pg/tube) and human IGF-1 antisera (Lot # AFP4892898, 1:400,000 final dilution). The antiserum had less than 1.0% cross-reactivity with human IGF-II. Amersham International (Arlington Heights, IL) supplied iodinated IGF-1 label ($IGF\text{-}1^{I125}$ sp act 2000 Ci/mmol; 16,000 cpm/tube) and Amerlex-M donkey anti-rabbit secondary antibody (code RPN510, 500 μL/tube). Buffer reagents were purchased from Fisher Chemical Co. (Pittsburgh, PA). Briefly, 20 μL plasma samples were aliquoted into polypropylene tubes, extracted with 400uL acid-ethanol, and incubated 30 min prior to centrifugation (2500×G; 4°C; 10 min). For each sample, supernatant (100 μL) was pipetted into duplicate polypropylene tubes and air-dried. IGF-1 standards were prepared in duplicate with 100 μL of known concentrations of human recombinant IGF-1 standard (ranging from 9 - 2500 pg/tube), and 300 L RIA buffer (200 mg/L protamine sulfate, 4.14 g/L sodium phosphate monobasic, 0.05% TWEEN 20, 0.02% sodium azide, 3.72 g/L EDTA) added to each tube. Air-dried samples were reconstituted with 350

μL RIA buffer and vortexed. To each sample was added 50 μL IGF-1 antibody (human IGF-1 antisera, UB3-189) at a 1:10,000 final dilution. After adding 100 μL of iodinated IGF-1 label (I^{125}-IGF-1), with ~15,000 CPM, samples were vortexed and incubated (4° C) overnight. Bound-free separation of IGF-1 was accomplished by incubating samples for 10 min with 500 μL of secondary antibody (Amerlex-M donkey anti-rabbit secondary antibody, code RPN.510 obtained from Amersham International) at a final dilution of 1:10,000. Sample tubes were centrifuged (2500×G; 4°C; 10 min) to separate the secondary antibody, which is bound to the primary antibody and ligand. The supernatant was decanted and the pellet counted on a Beckman 5500B gamma counter. Plasma samples were run in 3 assays having an average intraassay variance of 3.65% and an interassay variance of 4.63%. Plasma dilutions were parallel to the standard curve for IGF-1 RIA (ANCOVA; $F = 0.67$, $P = 0.43$; Fig. 3-4C).

Statistics

Tissue mass is typically highly correlated to body mass; thus, tissue weights were compared among months using ANCOVA, with body mass as a covariate, followed by Fishers Protected LSD post hoc. Data were presented as adjusted mean mass (mg) ± SEM. Pair-wise monthly comparisons, of mature and immature ovary stages, was performed with non-parametric chi-square analyses. Concentrations of E_2, T, and IGF-1 were estimated from raw data using the commercially available Microplate Manager software (Microplate Manager III, BioRad Laboratories, Inc., Hercules, CA, 1988). For RIA validation of pooled plasma dilutions and internal standards, hormone concentrations were log10-transformed prior to testing for homogeneity of slopes with standard curves by ANCOVA. Hormone concentrations of E_2, T, and IGF-1 were compared among months with one-way ANOVA followed by SNK post hoc contrasts. Tamhane post-hoc contrasts were used where variances were unequal among months for IGF-1 concentrations. The relationships between plasma hormones, tissue weights, air and water temperature, and rainfall were tested using Pearson's correlation analysis. Statistical

analyses were performed using SPSS software (v. 10, SPSS Inc., Chicago, IL, 1999) with $\alpha = 0.05$.

Results

Seasonal Environmental Parameters

Elevated air temperatures on Orange Lake between March and July of both years overlapped with the reproductive period of *R. grylio*, as determined by patterns of peak plasma E_2 and T concentrations described below. Conversely, decreased air temperatures overlapped with the non-reproductive period between November and February (Fig. 3-5). High levels of precipitation between June and September of 2002 overlapped the reproductive period season but rainfall fluctuated considerably throughout 2003. Water temperature, pH, and nitrate and nitrite ion concentrations were recorded between December of 2002 and July of 2003. Water temperature was low between December and January, and showed a steady increase in February that continued through the 2003 reproductive season (Fig. 3-5). Water pH between December and May ranged from 6.5 to 6.8 and between June and July ranged from 5.7 to 6.0. Aquatic nitrate and nitrate concentrations remained below 1 mg/L throughout the 2003 season. Generally, peak reproductive condition, as determined by reproductive tissue weights and plasma E_2 and T concentrations, was considered to occur between April and July of 2002 and between March and May of 2003, indicating that reproductive condition in *R. grylio* occurred during different months over the 15 month study.

Seasonal Tissue Mass and Ovarian Maturation

Fat body weights exhibited seasonal variation with the greatest weights occurring during June of 2002 and during January and March of 2003. The lowest fat body weights occurred between July and December of 2002 and between April and July of 2003 (Fig. 3-6A). Liver weights, which varied comparatively less with season, were greatest in April and March, and lowest between September and December of 2002 (Fig. 3-6B). Oviductal weights were greatest between April and July of 2002 and in May of 2003, whereas lowest weights occurred between

August of 2002 and March of 2003. Oviductal weights were also low between June and July of 2003 (Fig. 3-6C). Ovarian weights (GSI) were greatest in June of 2002, intermediate in May of 2002 and between March and May of 2003, and were lowest in April and between July and December of 2002, in addition to in June and July of 2003 (Fig. 3-6D).

A distinct seasonal pattern of ovarian maturation stages was observed in *R. grylio* (Fig. 3-3, 3-7). Frogs with black ovaries were considered to be in reproductive condition. Thus, frogs having either black or mature ovaries were considered reproductively mature for analyses. Conversely, frogs having either regressed or yellow ovaries, indicative of immature ovarian follicles, were considered reproductively immature for analyses. A greater percentage of frogs collected during the reproductive period had reproductively mature ovaries (Fig. 3-10). On average, approximately 80% of the females examined during the reproductive period had reproductively mature ovaries. In contrast, between 50% - 80% of frogs collected during the non-reproductive period (August - December) had reproductively immature ovaries (Fig. 3-7) but not all females collected had regressed ovaries this period.

Seasonal Plasma Steroid and IGF-1 Concentrations

Plasma E_2 concentrations were elevated during the reproductive period of both years compared to the non-reproductive period. Additionally, E_2 concentrations were higher in 2002 than in 2003 (Fig. 3-8A). Plasma T concentrations were elevated during the reproductive period of both years compared to the non-reproductive period. However, the period of elevated T concentrations was of slightly shorter duration in 2002 than in 2003 (Fig. 3-8B). Plasma IGF-1 concentrations exhibited a pattern opposite that of T between reproductive periods. Plasma IGF-1 concentrations were increased during both reproductive period compared to the non-reproductive period. However, IGF-1 concentrations were elevated for more months in 2003 than in 2002 (Figure 3-8C). The variation in environmental factors associated with variation in plasma hormone concentrations between the two reproductive periods indicate that reproductive physiology of *R. grylio* is influenced by environmental factors.

Throughout the season, plasma E_2 concentrations were comparatively lower than plasma concentrations of T and IGF-1. Plasma E_2 and IGF-1 concentrations peaked during similar months but plasma E_2 concentrations declined precipitously after the reproductive period whereas plasma IGF-1 declined less sharply and remained elevated slightly longer. A peak in plasma T concentration occurred slightly prior to increases in plasma concentrations of E_2 and IGF-1 (Fig. 3-9).

Peaks in ovarian and oviductal weights generally corresponded to elevated plasma T and E_2 concentrations, but they paralleled plasma T concentrations more closely (Fig. 3-15). During the non-reproductive period, plasma steroids, plasma IGF-1, and reproductive tissue weights were lower than in the reproductive period. Plasma IGF-1 concentrations appeared to peak in the latter months of the reproductive period after steroid concentrations and the weights of reproductive tissues began to decline (Fig. 3-10). Liver and fat body weights increased before, or in association with, peaks in plasma T concentrations and much earlier than peaks in E_2 and IGF-1 concentrations (Fig. 3-11).

Correlations: Plasma Steroids, Tissue Mass, and Environmental Parameters

For some months, the sample number of frogs collected was small; thus, analyses were focused on correlations among means for all months. A strong positive correlation was detected between plasma E_2 and T concentrations ($r^2 = 0.67$; $P = 0.006$) but not between concentrations of E_2 and IGF-1 and not between IGF-1 and T. Plasma E_2 concentration correlated strongly to oviductal weights ($r^2 = 0.84$; $P < 0.0001$), to ovarian weights ($r^2 = 0.51$; $P < 0.05$), and to water temperature ($r^2 = 0.71$; $P = 0.05$). Plasma T concentrations correlated strongly with ovarian ($r^2 = 0.85$; $P < 0.0001$) and oviductal ($r^2 = 0.76$; $P = 0.001$) weights but not to environmental parameters. Plasma IGF-1 concentrations were negatively correlated to air temperature ($r^2 = 0.55$; $P = 0.03$) and positively correlated with fat body weights ($r^2 = 0.62$; $P = 0.02$) but not correlated to other parameters. Additionally, correlations were detected between ovarian and oviduct ($r^2 = 0.59$; $P = 0.02$), or fat body masses ($r^2 = 0.57$; $P = 0.03$), and between oviductal and liver weights

($r^2 = 0.71$; $P = 0.003$). Finally, fat body weight was correlated with water temperature ($r^2 = 0.75$; $P = 0.03$; Table 3-2).

Discussion

Relatively few studies have described a pattern of seasonal changes in the reproductive tissues obtained from wild populations of ranid frogs (Ligas, 1960; Licht et al., 1983; Kim et al., 1998). Previous studies of reproductive cyclicity in *R. grylio* have been limited to ovarian maturation, male calling behavior, sexual dimorphism, and observations of amplexus (Lygas, 1960; Lamb, 1983; Wood et al., 1998). Licht et al. (1983) reported that ovarian and oviductal weights in *R. catesbeiana* increased during the reproductive season, between May and July, declined sharply in August, and remained reduced through October in a population from central California.

In *R. grylio* from Orange Lake, increased ovarian and oviductal weights and plasma steroid concentrations clearly define the months during which peak reproductive condition occurred during this study. Analysis of ovarian maturation stages revealed that the largest percentage of frogs had mature ovaries during the reproductive period between April and July of 2002 and between March and July in 2003. During the months of the non-reproductive period, a greater percentage of frogs had regressed ovaries. A similar pattern of ovarian maturation was described for *R. grylio* in the Okefenokee Swamp of Georgia and in the Everglades of South Florida (Ligas, 1960; Lamb, 1983).

The reproductive period occurred during slightly different months over the 15 month study, lasting between April and July of 2002 but only between March and June of 2003. Our data indicate that reproductive condition of *R. grylio*, as in other amphibians studied previously, is responsive to changing environmental conditions. The reproductive period generally overlapped with the months of high water and air temperature, and of high rainfall during both years. Also, concentrations of plasma IGF-1 increased with rising air temperature. Reproductive tissue weights were greatest during periods of elevated air and water temperature, but no

significant correlations were observed between tissue weights and air or water temperature. However, fat body weights decreased with increasing air temperature. These data lend support to the theory that fat bodies are an energy reserve that are metabolized at the onset of warmer weather to fuel rapid growth of reproductive tissues for breeding activity. Additionally, fat body weights were positively correlated to ovary weights. Saidapur and Hoque (1995) reported similar findings for *Rana tigrina* in India where decreasing fat body weights corresponded to increased egg production, and both fat body weights and egg production were correlated to increasing air temperature. In *R. grylio* from the Florida Everglades, reproductive activity is reportedly suppressed during periods of low air and water temperature, and cease entirely during periods of drought (Lygas 1960). The reproductive period of these frogs occurred primarily between March and September and the non-reproductive period extends from October to February. Unlike frogs from Orange Lake, *R grylio* from the Everglades appear to have an extended reproductive period based on the mature ovarian tissue late into the season. This is supported by observations of calling behavior by males, which also extends late into the season. However, it was unknown if female *R. grylio* in the Everglades were actively mating and ovipositing eggs during these times, so the extended reproductive period is speculative. This temporal variation in reproductive period according to season could be attributed geographic differences, extended months of warm temperatures in the summer, and milder temperatures during the winter in the Everglades compared to north-central regions of Florida. Licht et al. (1983) attributed a similar temporal variation in reproductive periods of bullfrogs according to geographic location. Also, in the Okefinokee Swamp of Georgia, male *R. grylio* continue vocalizing between March and September but peak reproductive condition of females occurs during June and July (Wright, 1932; Wright and Wright, 1949). These studies indicate that reproductive periods for *R. grylio* are associated with localized environmental changes and also with geographic location.

The increase in relative liver weights of *R. grylio*, just prior to the onset of the reproductive season, is indicative of increased hepatic biochemical or secretory activity. The liver

synthesizes many proteins that regulate metabolism, growth, reproduction, and development. One of these proteins, vitellogenin, is a precursor of egg yolk in oviparous ectotherms and provides valuable nutritive and energetic support for developing embryos (Carnevali et al., 1995; Sumpter and Jobling, 1995; Guillette et al., 1996; Palmer and Guillette, 1998). Estrogen produced by mature ovaries stimulates vitellogenesis in the liver. Vitellogenin is a yolk precursor protein that is transported through the plasma to the ovaries where it accumulates within developing ova (Licht, 1979)(Palmer et al., 1998; Sumpter and Jobling, 1995). In female alligators vitellogenesis is accompanied by an elevation of plasma IGF-1 concentrations during the reproductive period. IGF-1 has also been detected in the egg albumin of birds and reptiles, suggesting that this hormone plays a role in embryonic growth and development (Cox and Guillette, 1993; Guillette et al., 1996). In *R. grylio*, liver weights were elevated before peaks in plasma IGF-1 concentrations, and might reflect hepatic synthesis of proteins that function in reproduction (Palmer et al., 1998; LeRoith et al., 2001b). In oviparous ectotherms, plasma IGF-1 is also influenced by nutritional status and feeding activity (Crain et al., 1995). In this study, plasma IGF-1 concentrations were negatively correlated to decreasing fat body weights and positively correlated to increasing air temperature. Accordingly, in *R. grylio*, the correlation between plasma IGF-1 concentrations and fat body weights might reflect metabolism of stored fat (during the warmer months of the spring and summer months) to provide energy for growth of reproductive tissues. In contrast, decreased concentrations of plasma IGF-1 during the non-reproductive period might reflect a decline in feeding behavior and in fat metabolism in female *R. grylio*.

Seasonal changes in concentrations of plasma steroids and IGF-1, in association with changes in reproductive tissues, are largely undescribed for anurans. Seasonal changes in reproductive tissues, and in concentrations of plasma steroids and plasma gonadotropins had been described in ranid frogs from temperate North American and in India (Licht et al. 1983, Kim et al., 1998). In California *R. catesbeiana*, plasma E_2 and T concentrations generally peaked between April and June; a pattern similar to that shown for plasma steroids in *R. grylio*. Plasma

steroid concentrations in *R. grylio* were most similar (1-4 ng/mL for E_2 and 20-80 ng/mL for T) to those measured in bullfrogs within 12 h of capture (Licht et al., 1983). Although plasma E_2 and T concentrations in *R. grylio* were positively correlated to each other, only plasma T concentrations were correlated to ovarian and oviductal weights. This observation conflicts with findings in mammals but indicates it might be common among non-mammalian vertebrates. In ectotherms, androgens might play an important role in regulating reproductive condition. Amphibian ovarian follicles synthesize and secrete large quantities of androgens during ovarian maturation (Fortune and Tsang, 1981; Fortune, J.E. 1983; Lutz et al., 2001). Androgens might be aromatized to estrogens in peripheral tissues such as the brain, fat and skin (Follett and Redshaw, 1968). The oviduct might also be a site of peripheral aromatization of androgens and be a target for androgen activity. The oviduct of oviparous species synthesizes huge quantities of protein (perhaps in response to androgen stimulation) for use as secondary or tertiary egg coatings such as in anuran egg jellies (Maack et al., 1985; Olsen and Chandler, 1999; Arranz and Cabada, 2000; Jesu-Anter and Carroll, 2001). Similar to *R. grylio*, female *R. catesbeiana* also exhibited greater T than E_2 plasma concentrations indicating that this pattern might be prevalent among ranids (Licht et al., 1983). *Rana grylio* exhibited peak ovarian and oviductal weights during similar months as reported for bullfrogs (Licht et al., 1983). However, plasma steroid concentrations in *R. grylio* did not decrease significantly 24 h after capture as reported for *R. catesbeiana* (Licht et al., 1983). In *R. catesbeiana*, increases in ovarian and oviductal mass closely paralleled increases in plasma gonadotropins and steroids (Licht et al., 1983). Plasma LH and FSH were not measured in *R. grylio* and it remains unknown whether plasma gonadotropins increased before elevations in plasma steroid concentrations or tissue mass. In future studies, it would be valuable to examine changes in plasma gonadotropins with respect to steroids to better understand the reproductive cycle of *R. grylio*.

Before this study on *R. grylio*, seasonal changes in plasma IGF-1 concentration were reported for only one other anuran species, *Bufo woodhousei* (Pancak-Roessler and Lee, 1990).

Plasma IGF-1 concentrations in *B. woodhousei* peaked (1 ng/ml) in July and declined sharply thereafter. In *R. grylio*, plasma IGF-1 concentrations peaked between May and July and declined after August. In *R. grylio* a peak in plasma IGF-1 concentrations occurred later in the season compared to *Bufo woodhousei* and is likely due to several factors including geographical variation, interspecific differences, and differences in IGF-1 RIA methods.

In reptiles, seasonal changes in plasma IGF-1 concentrations have been described for alligators and turtles. In loggerhead sea turtles, elevated plasma IGF-1 concentrations occurred between April and June and were associated with reproductive activity and increased feeding behavior of female turtles during these months (Crain et al., 1995). Guillette et al. (1996) examined reproductive tissues, and plasma steroids and plasma IGF-1 concentrations, and their respective associations with reproductive condition in alligators. In female alligators, plasma IGF-1 concentrations increased in June and were associated with gravidity. Elevated plasma E_2 and P_4 concentrations were associated with peak vitellogenesis, and also preceded gravidity and peaks in plasma steroid concentrations. Seasonal patterns of plasma IGF-1 concentrations were not examined simultaneously with changes in plasma steroids concentrations; therefore, it is unknown how these hormones change (with respect to each other) seasonally in alligators (Guillette et al., 1996). In a separate study, alligators collected from the same locality exhibited a peak in plasma E_2, T, and P_4 concentrations in May (Guillette et al., 1997). Thus, alligators are similar to *R. grylio* in that elevated plasma steroid concentrations precede peaks in plasma IGF-1.

In mammals, IGF-1 expression is associated various aspects of reproduction including ovarian maturation, follicular atresia, selection of dominant follicles, and regulation of gonadal steroidogenesis. Increasing concentrations of IGF-1 can be synthesized in reproductive tissues or the in liver, and can be transported directly to offspring in utero. In contrast to mammals, oviparous animals must provide growth-promoting substances to eggs prior to oviposition (Palmer and Guillette, 1991; Guillette et al., 1996). Accordingly, IGF-1 has been detected in the

yolks of chicken eggs, the albumen of alligator eggs, and the oviductal glands of geckos and alligators (Scavo et al., 1989; Guillette and Williams, 1991; Cox and Guillette, 1993; Cox, 1994).

In conclusion, this study provides the first evidence that IGF-1 is present in the plasma of *R. grylio*. Plasma IGF-1 concentrations were correlated with several environmental factors and exhibited a clear pattern of change with reproductive period, and with reproductive steroid concentrations and weights of reproductive tissues. Although the role of IGF-1 in anurans requires further study, this study has provided valuable information for understanding the association of IGF-1 with reproductive physiology in *R. grylio*.

Table 3-1. Comparison of plasma insulin-like growth factor-1 (IGF-1) concentrations among mammalian, avian, reptilian, and amphibian species.

	IGF-1 (ng/mL)	Reference
Mammalian		
Rat		
Juvenile males	574.0	a
Human		
Adult females (Reproductive status unknown)	261.0	a
Cow		
Adult females (Reproductive status unknown)	182.0	a
Avian		
Chicken		
Juveniles (8-week)	42.0	a
Reptilian		
Red-eared slider turtle		
Juvenile males	17.0	a
Loggerhead sea turtle		
Reproductive	7.5	b
Non-reproductive	3.0	b
American alligator		
Reproductive	16.0	c
Non-reproductive	5.0	c
Amphibian		
African Clawed frog		
Non-reproductive females	3.0	d
American toad		
Reproductive males	4.0	d
Non-reproductive males	0.5	
Bullfrog		
Non-reproductive female	1.0	d
Marine toad		
Non-reproductive female	1.0	d
Pig frog		
Reproductive females	22.0	This Study
Non-reproductive females	10.0	This Study

a - Daughaday et al., 1985
b - Crain et al., 1995
c - Guillette et al., 1996
d - Pancak-Roessler and Lee, 1990
Note - For all studies shown, plasma IGF-1 was measured, after acid-extraction of IGF-1 binding proteins, by radioimmunoassay.

Table 3-2. Correlations among body mass, snout vent length (SVL), hormone concentrations, tissues weights of *Rana grylio*, and environmental parameters.

Pearson Correlation	P-value	R^2	Relationship
Body Mass and E_2	0.27		
Body Mass and T	0.06		
Body Mass and IGF-1	0.94		
Snout-Vent-Length and E_2	0.31		
Snout-Vent-Length and T	0.09		
Snout-Vent-Length and IGF-1	0.58		
Plasma E_2 and T	0.006 **	0.67	+
Plasma E_2 and IGF-1	0.28		
Plasma E_2 and Ovary Weight	0.05	0.51	+
Plasma E_2 and Oviduct Weight	< 0.0001 **	0.84	+
Plasma E_2 and Liver Weight	0.18		
Plasma E_2 and Fat Body Weight	0.88		
Plasma E_2 and Air Temperature	0.07		
Plasma E_2 and Water Temperature	0.05	0.71	+
Plasma E_2 and Rainfall	0.77		
Plasma T and IGF-1	0.64		
Plasma T and Ovary Weight	< 0.0001 **	0.85	+
Plasma T and Oviduct Weight	0.001 **	0.80	+
Plasma T and Liver Weight	0.06		
Plasma T and Fat Body Weight	0.16		
Plasma T and Air Temperature	0.20		
Plasma T and Water Temperature	0.09		
Plasma T and Rainfall	0.25		
Plasma IGF-1 and Ovary Weight	0.81		
Plasma IGF-1 and Oviduct Weight	0.67		
Plasma IGF-1 and Liver Weight	0.35		
Plasma IGF-1 and Fat Body Weight	0.02 *	0.62	–
Plasma IGF-1 and Air Temperature	0.03 *	0.55	+
Plasma IGF-1 and Water Temperature	0.12		
Plasma IGF-1 and Rainfall	0.48		
Ovary and Oviduct Weight	0.02 *	0.59	+
Ovary and Liver Weight	0.25		
Ovary and Fat Body Weight	0.03 *	0.57	+
Oviduct and Liver Weight	0.003 **	0.71	+
Oviduct and Fat Body Weight	0.27		
Liver and Fat Body Weight	0.13		

Table 3-2. Continued.

Pearson Correlation	P-value	R^2	Relationship
Air Temperature and Ovary Weight	0.84		
Air Temperature and Oviduct Weight	0.37		
Air Temperature and Liver Weight	0.70		
Air Temperature and Fat Body Weight	0.08		
Water Temperature and Ovary Weight	0.45		
Water Temperature and Oviduct Weight	0.07		
Water Temperature and Liver Weight	0.59		
Water Temperature Fat Body Weight	0.03 *	0.75	–
Rainfall and Ovary Weight	0.55		
Rainfall and Oviduct Weight	0.88		
Rainfall and Liver Weight	0.37		
Rainfall and Fat Body Weight	0.84		

* Significant
** Highly Significant
\+ positive correlation
– negative correlation

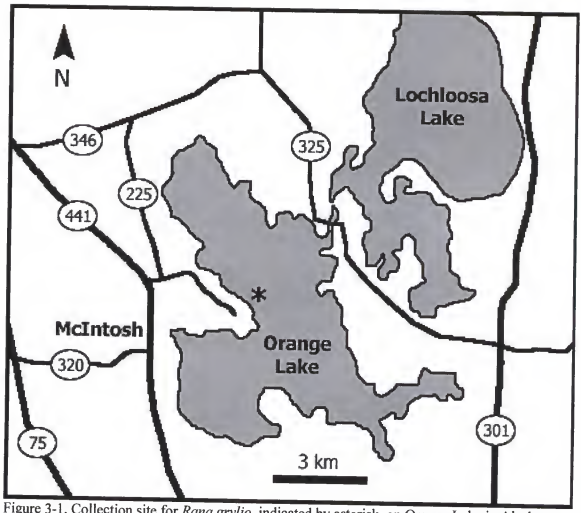

Figure 3-1. Collection site for *Rana grylio*, indicated by asterisk, on Orange Lake in Alachua county, Florida (Latitude 29°27.853'N, Longitude 82°11.380'W) between April, 2002 and July, 2003. Image created by T. Barbeau.

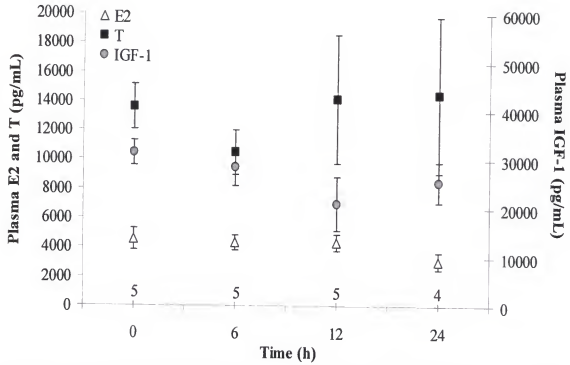

Figure 3-2. Plasma 17β-estradiol (E_2), testosterone (T), and insulin-like growth factor-1 (IGF-1) concentrations in *Rana grylio* at 0, 6, 12, and 24 h post-capture. Plasma samples for each time interval were collected from different frogs. Data presented as means ± SEM. Letters within axes represent sample size at each time interval. No significant differences detected among time intervals for each hormone (ANOVA; E_2 $P = 0.40$;T $P = 0.83$; IGF-1 $P = 0.42$).

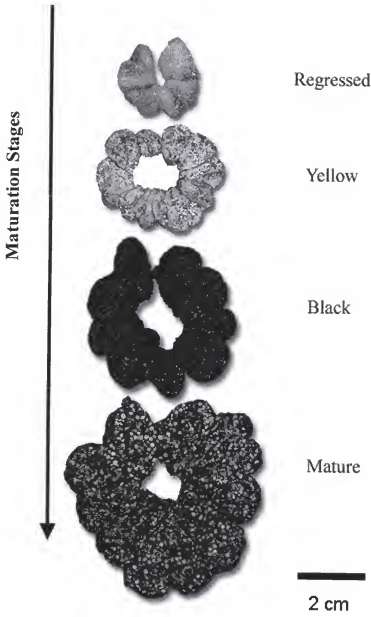

Figure 3-3. Staging of *Rana grylio* ovaries in progression from least to most mature stages. A. regressed (stage 1), B. yellow (stage 2), C. black (stage 3), and D. black and white (stage 4) ovaries.

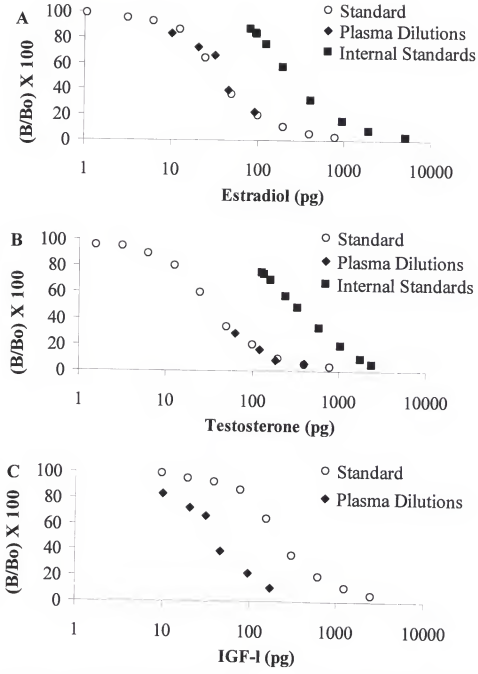

Figure 3-4. Biochemical validation of *Rana grylio* plasma for RIA. A. For 17β-estradiol RIA internal standard and plasma dilution curves were parallel to the standard curve (ANCOVA; $F = 0.24$, $P = 0.63$ and $F = 2.89$, $P = 0.15$). B. For testosterone RIA internal standard and plasma dilution curves were parallel to the standard curve (ANCOVA; $F = 0.001$, $P = 0.99$ and $F = 0.01$, $P = 0.92$). C. For insulin-like growth factor-1 (IGF-1) RIA the plasma dilution curve was parallel to the standard curve (ANCOVA; $F = 1.05$, $P = 0.33$).

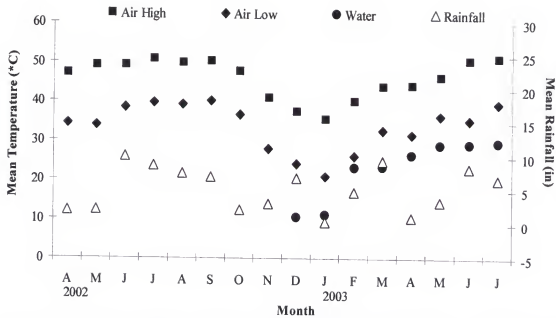

Figure 3-5. Monthly changes in rainfall, water temperature, and high and low air temperature at collection site on Orange Lake, Florida. Data presented as means per month.

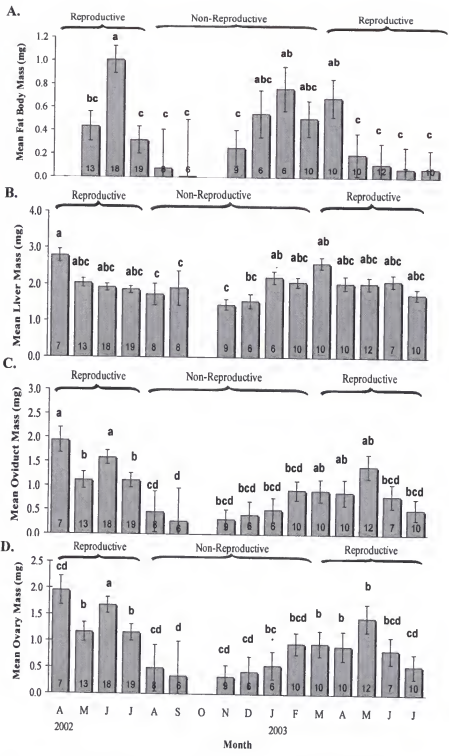

Figure 3-6. Seasonal change in fat body weights in *Rana grylio* during the reproductive and non-reproductive periods. Data presented as means ± SEM. Numbers within bars indicate sample size and different letters above bars indicate significantly different means A. for fat bodies (ANCOVA; $F = 2.78$, $P = 0.002$), B. for liver (ANCOVA; $F = 4.90$, $P < 0.001$), C. for oviducts (ANCOVA; $F = 1.55$, $P < 0.001$), and D. ovaries (ANCOVA; $F = 4.27$, $P < 0.001$).

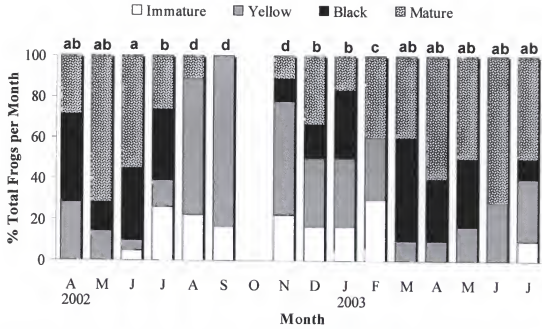

Figure 3-7. Seasonal changes in ovarian maturation stages of *Rana grylio*. Data presented as percentage of frogs exhibiting immature, yellow, black, and mature ovary stages, out of total frogs, from the total collected that month. Different letters above bars indicate significantly different percentages as determined by Mann Whitney U pairwise contrasts ($P < 0.05$).

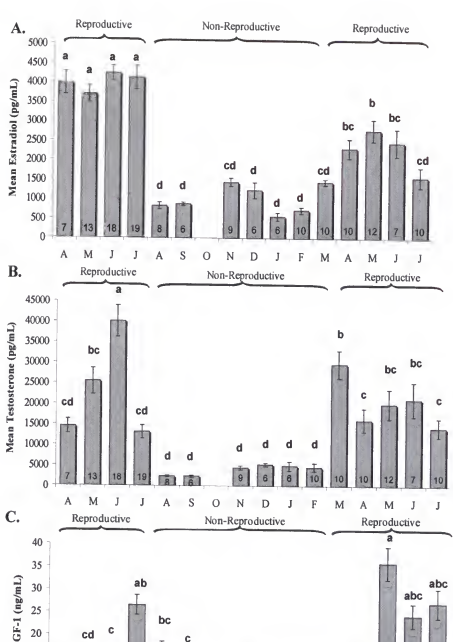

Figure 3-8. Seasonal change in plasma hormones in *Rana grylio* during the reproductive and non-reproductive periods. Data presented as means ± SEM. Numbers within bars indicate sample size while different letters above bars indicate significantly different means for A. 17β-estradiol (ANOVA; $P < 0.001$), B. testosterone (ANOVA; $P < 0.001$), and C. insulin-like growth factor-1 (IGF-1, ANOVA; $P < 0.001$).

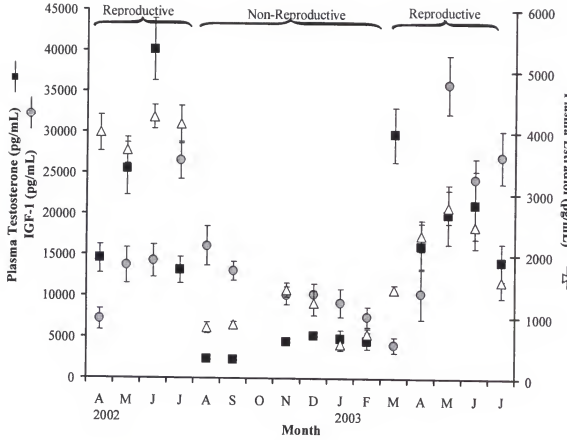

Figure 3-9. Seasonal changes in plasma 17β-estradiol (E_2), testosterone (T), and insulin-like growth factor-1 (IGF-1) in *Rana grylio* during the reproductive and non-reproductive periods. Data presented as means ± SEM.

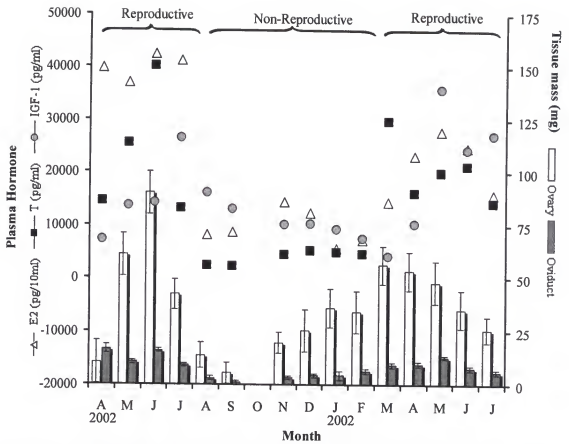

Figure 3-10. Seasonal changes in plasma 17β-estradiol (E_2), testosterone (T), and insulin-like growth factor-1 (IGF-1), and of ovary and oviduct weights in *Rana grylio* during the reproductive and non-reproductive periods. Steroid data presented as means and tissue data presented as means ± SEM.

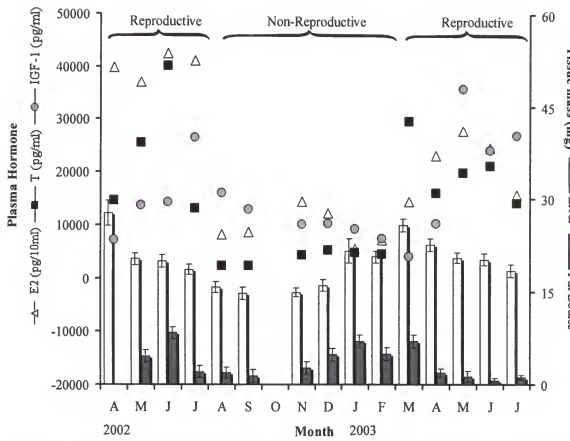

Figure 3-11. Relative seasonal changes in plasma 17β-estradiol (E_2), testosterone (T), insulin-like growth factor-1 (IGF-1), and of liver and fat body weights *Rana grylio* during the reproductive and non-reproductive periods. Steroid data presented as means and tissue data presented as means ± SEM.

CHAPTER 4
THE EFFECTS OF INSULIN-LIKE GROWTH FACTOR-1 AND ESTRADIOL IMPLANTS (*IN VIVO*) ON OVIDUCT MORPHOLOGY, AND ON PLASMA HORMONES IN BULLFROGS (*Rana catesbeiana*)

Introduction

The regulation of oviduct growth and function by endocrine hormones has been described for mammals and some reptiles but comparatively little is known for amphibians (Christiansen, 1973; Mead et al., 1981; Murphy and Ghahary, 1990; Cox and Guillette, 1993; Buhi et al., 1999; Girling et al., 2000). Most studies of frog reproduction have focused on variation in plasma concentrations of steroid hormones and ovarian maturation, with little attention to regulation of oviductal structure (Licht et al., 1984; Wake and Dickie, 1998). For oviparous animals, the oviduct is vital for reproduction because it synthesizes and secretes important substances that nourish and encapsulate ovulated oocytes. The female bullfrog (*Rana catesbeiana*) can oviposit as many as 40 to 80 thousand eggs at one breeding event (Norris, 1997). Without the provision of oviductal secretions, oocytes could not be fertilized successfully nor could they develop into normal embryos (Low et al., 1976; Buhi et al., 1997; Buhi et al., 1999; Olsen and Chandler, 1999). The amphibian oviduct includes four major structural and functional regions: the infundibulum, the atrium, the ampulla, and the ovisac (Uribe et al., 1989). The infundibulum is the anterior-most region of the oviduct and receives mature oocytes ovulated from the ovaries. Distal to the infundibulum is the atrium - a narrow aglandular region that precedes the ampulla. The ampulla region is longest portion of the oviduct and contains numerous glands within the endometrial layer (Wake and Dickie, 1998). The glands within the ampulla region are biochemically active and secrete a variety of substances that are incorporated into mature oocytes as they traverse the oviduct (Uribe et al., 1989). The last region of the oviduct is the ovisac or

uterus that leads to the cloaca. The narrow and aglandular ovisac is the final site from which oocytes are deposited from the reproductive tract into the environment.

Oviductal growth occurs primarily in response to stimulation by elevated E_2 concentrations of E_2, of ovarian origin, and involves proliferation of epithelial and endometrial cells (Christiansen, 1973; Mead et al., 1981; Cox, 1994). In amphibians, the major reproductive steroids progesterone (P_4), testosterone (T), and 17β-estradiol (E_2), are produced and secreted by the ovary, in response to pituitary follicle stimulating hormone (FSH) and luteinizing hormone (LH) (Licht, 1979; Chapter 3). The principal steroid that regulates structure and function of the oviduct is reported to be E_2. In addition to E_2, polypeptide growth factors have been shown to elicit a growth response in the reptilian and mammalian oviduct (Cox and Guillette, 1994; Stevenson et al., 1994; Tang et al., 1994; Richards et al., 1997). Growing evidence demonstrates that autocrine and paracrine sources of epidermal growth factor (EGF) and insulin-like growth factor-1 (IGF-1) are potent hormonal mitogens that mediate E_2-induced oviduct growth. In mammals, these growth factors induce oviduct growth in the absence of endogenous E_2, and induce an even greater growth in the presence of E_2 compared to either hormone administered alone (Nelson, 1991; Murphy and Murphy, 1994). These findings indicate a hormonal synergy between E_2 and IGF-1 in the stimulation of oviduct growth.

Cox and Guillette (1994) reported that ovariectomized geckos exhibit oviductal growth in response to EGF and IGF-1 even in the absence of stimulation by endogenous E_2. However, neither EGF nor IGF-1 stimulation induced oviduct growth similar to that observed with E_2 alone. Unfortunately, the effect of simultaneous treatment with E_2 and growth factors on oviduct growth was not examined in this study and it remains unknown if an E_2 and IGF-1synergy exists for these animals.

In this study, I examined the effects of controlled doses of steroid hormone (E_2), and peptide hormones (IGF-1 and EGF) on oviduct growth in adult, female bullfrogs *(Rana catesbeiana)*. The objective of this study was to determine whether the oviducts in *R. catesbeiana*

exhibited a growth response with exposure to E_2 or to growth factors administered separately or in combination. I predicted that ovariectomized *R. catesbeiana* treated with either E_2 or growth factors (EGF and IGF-1) would exhibit oviduct growth. Additionally, I predicted that oviduct growth would be greater in frogs treated simultaneously with E_2 and IGF-1 than with either hormone alone.

Materials and Methods

Adult female *Rana catesbeiana* (N = 65) were purchased (Charles D. Sullivan Co. Inc., TN). They were maintained under a 12-h diurnal light/dark cycle in 38 L tanks with 19 L of static, dechlorinated water at 26ºC. They were fed crickets every other day throughout the experiment. Frogs were randomly assigned to each of the following treatment groups: E_2 (N=10), IGF-1 (N=10), EGF (N=10), E2 /IGF-1 (N=10), placebo (N-10), and sham (N=5). The use of sham frogs is explained below. Weight and snout vent length (SVL) of frogs were recorded and no significant difference was detected in mass (ANOVA; $P = 0.84$) or SVL (ANOVA; $P = 0.85$) of frogs among treatment groups. Numbered stainless steel tags were applied to the webbing of the hind foot of frogs for individual identification. Animals were maintained and experiments were performed as approved by the Institute for Animal Care and Use Committee (IACUC project #Z095).

Sham animals are frogs that were subjected to identical anesthesia and surgical procedures that ovariectomized frogs were subjected to (explained below) with the exception that the ovaries were not removed and they received no hormone treatment. Thus, sham frogs were intact frogs that were included to account for an effect of the surgical procedure itself on physiological responses of the frogs.

For this study, *R. catesbeiana* were chosen in lieu of *X. laevis* and *R. grylio* that were examined in earlier chapters. Previous attempts to maintain wild-caught *R. grylio* in captivity demonstrated that this species exhibited considerable stress and was considered inappropriate for a long-term, surgical study. In contrast to *R. grylio*, *R. catesbeiana* were very adaptable to

captivity and exhibited less stress. Previous attempts to ovariectomize *X. laevis* were largely unsuccessful while *R. catesbeiana* responded optimally to the ovariectomy procedures with low mortality and fast recovery. Additionally, *R. catesbeiana* are large-bodied frogs and it was easier to collect blood samples of greater volume than in *X. laevis* and *R. grylio*. Lastly, *R. catesbeiana* is closely related to *R. grylio* and was considered a relevant and appropriate substitution for this study.

Ovariectomy

After a 2-week acclimation period, frogs were anesthetized with MS-222 (1.5% 3-aminobenzoic acid ethyl ether, Aquatic Ecosystems, Orlando, FL), and ovariectomy was performed. The ovaries were removed to ensure that endogenous hormones did not conflict with or obscure effects observed in response to experimental treatments. Conducting more than six surgeries per day would have compromised my ability to carefully conduct surgeries and oversee post-operative recovery of individuals. Thus, ovariectomy was performed each day, for 10 consecutive days, on one individual selected from each of the six treatment groups.

Under sterile conditions, a 2.5 cm right paramedial incision was made through the skin and muscle layers into the abdominal cavity, and the left and right ovaries were excised through this single incision. Hemostasis of the mesovarium (vascular tissue supporting the ovaries) was accomplished by a series of double-ligatures of the vessels using 5-0, monofilament nylon suture material (Fig. 4-1). The incision layers (peritoneal, muscle, and skin) were closed with a single interrupted pattern using the same suture material. For each female, the mass of excised ovaries was recorded and reproductive status of the female was determined by visual inspection of ovarian follicle maturation according to Dumont (1971). This procedure was performed to confirm that females were reproductively similar at the onset of the experiment to minimize variation in responses to subsequent treatments.

Pre-ovariectomy blood samples were collected by cardiac puncture to determine whether plasma E_2, T, and IGF-1 concentrations were similar among females at the start of the

experiment. No more than 1.0% of total blood volume estimated per body mass was taken from frogs (Mader, 1996; Wright, 2001). Blood samples were stored in heparin vacutainer tubes, centrifuged, and subsequent plasma was stored (-70°C) for radioimmunoassay (RIA) analyses. If blood could not be collected within two cardiac punctures attempts were ceased to avoid potential injury to the frog. Blood could not be sampled from all individuals prior to surgery; therefore, sample sizes for pre-ovariectomy blood samples were as follows: E_2 (N= 8), IGF-1 (N=9), EGF (N=7), E2 /IGF-1 (N=9), placebo (N=8), and sham (N=5).

Post-ovariectomy frogs were placed in recovery tanks containing benzalkonium chloride (antibiotic) dissolved in 1 liter of water for 48 h. Afterwards, recovered frogs were returned to their tanks. Frogs were allowed a 3-week recovery period during which the surgical sites were closely monitored for signs of inflammation or infection. Although most frogs experienced no post-operative complications, five frogs failed to recover from the ovariectomy. Therefore, the final sample sizes for post-ovariectomy treatment groups were as follows: E_2 (N=10), IGF-1 (N=10), EGF (N=8), E_2/IGF-1 (N=9), placebo (N=8), and sham (N=5). No further losses occurred, and these sample sizes were maintained for each treatment group for the remainder of the study.

Hormone Implants

After the 3-week recovery period, all frogs exhibited complete healing of the ovariectomy incision site (Fig. 4-2). Treatments were administered by surgical insertion of an intra-abdominal, 21-day release treatment pellet (Innovative Research of America, Sarasota, FL) containing either of the following dosages: E_2 (420 µg), IGF-1 (10 µg), EGF (10 µg), E_2/IGF-1 (420 µg E_2 and 10 µg IGF-1), and placebo (10 µg vehicle pellet). Surgical procedures for the treatment pellet implantation were similar to those described for ovariectomy with respect to incision and abdominal closure; however, an 1.0 cm left paramedial incision was made. Pellets were inserted into the abdominal cavity midway between the left and right oviducts (Fig. 4-3).

The E_2 treatment served as a positive control, while the placebo treatment served as a negative control. The EGF, IGF-1, and E_2/IGF-1 treatments were experimental. For simultaneous treatment with E_2/IGF-1, one pellet of each hormone was inserted 2.54 cm apart from each other within the abdomen. The hormone concentrations administered were physiologically relevant and chosen based on a literature review of similar studies in which treatments were given for durations ranging from 7 - 20 days to elicit a tissue response (Redshaw et al., 1968; Follet and Redshaw 1968; Fortune 1981; Cox 1994; Crain et al. 1995). After 18 days of treatment, the frogs were euthanized and examined as described below.

Tissue Sampling

The response of *R. catesbeiana* to 18 days of treatment was determined by measuring the following parameters: weights of tissues (liver and oviduct), oviductal growth (macroscopic and microscopic), and plasma concentrations of E_2, T, and IGF-1. After treatment, frogs were anesthetized with MS-222, and blood samples were collected. Frogs were euthanized by dissection through the spinal cord followed by pithing. Plasma samples (post-treatment) were frozen (-70°C) for RIA analyses. The liver and oviducts were removed from each frogs and weighed for comparison of wet tissue mass among treatment groups. Cross-sectional samples of oviducts were fixed in 4% paraformaldehyde (4°C; 48 h) followed by rinse and storage in 75% ethanol for subsequent histological analyses. The oviducts were dehydrated in a graded series of ethanol changes, embedded in paraffin, serially sectioned on a rotary microtome (7 μm), stained with modified Masson's staining procedure, and examined microscopically. To evaluate oviductal growth, an ocular micrometer was used to record 10 morphological measurements on 5 tissue sections per frog (for a total of 50 measures) for each of the following oviductal parameters: epithelial cell height, endometrial layer thickness, endometrial gland height, and endometrial gland width. The gland height and width measurements were used to calculate cross-sectional gland surface area.

Steroid Radioimmunoassay (RIA) Biochemical Validation

Validation samples were obtained by pooling plasma aliquots from each individual. Two methods were used to biochemically validate the E_2 and T RIA: internal standards and plasma dilutions. One half of the plasma pool, for use with internal standards, was mixed with Norit charcoal (10 mL plasma:1 g charcoal; 4°C; 24 h) to strip steroid hormones from the plasma. The solution was then centrifuged (3000 rpm; 1200×G; 45 min) and the resultant supernatant decanted. Separate aliquots of stripped plasma (25 μL) were added to 10 tubes and spiked with 100 μL of 1.56, 3.13, 6.25, 12.5, 25, 50, 100, 200, 400, 800 pg cold E_2 or T hormone. These tubes were extracted twice with ethyl-ether, air-dried, and reconstituted in 100 μL of borate buffer (100 μL; 0.05 M; pH 8.0).

For plasma dilutions, 6.25, 12.5, 25, 50, 100, and 200 μL plasma was added to 6 tubes. Appropriate volumes of borate buffer were added to each tube to bring the final sample volume to 200 μL. Samples were extracted twice with ethyl-ether, air-dried, and reconstituted with 100 μL of borate buffer. Resultant samples for both internal standards and plasma dilutions were examined by the RIA procedure described below.

Plasma extraction efficiencies were determined by adding 100 μL tritiated E_2 and T (15,000 cpm) to 100 μL of pooled plasma samples, twice extracting with ethyl-ether, air-drying, adding 500 μL scintillation fluid to tubes, and reading samples on a Beckman LS 5801 scintillation counter to determine the tritiated hormone remaining. The extraction efficiencies for E_2 and T samples were 93.9% and 87.9%, respectively. Supernatant (500 μL) was added to 5 mL of scintillation fluid, and counted on a Beckman scintillation counter. Plasma intraassay variance for E_2 and T validation RIAs for averaged 1.53% and 1.23%, respectively. Plasma interassay variance for E_2 and T averaged 2.87% and 4.88%, respectively.

Steroid RIA Procedures

RIAs were performed for E_2 and T on plasma samples collected both pre-ovariectomy and post-treatment. For pre-ovariectomy E_2 samples, 50 μL of plasma was used, and for post-treatment samples, 50 μL of plasma was used for E_2, E_2/IGF-1, and sham samples and 300 μL plasma used for IGF-1, EGF, and placebo samples. For pre-ovariectomy T samples, 30 μL of plasma was used while for post-treatment samples, 50 μL of plasma was used for E_2, E_2/IGF-1, and sham samples and 300 μL plasma used for IGF-1, EGF, and placebo samples. These volumes were selected for analysis based on RIA volume determinations conducted on these samples previously. Briefly, duplicates of plasma samples were twice extracted with ethyl ether, air-dried, and reconstituted in borate buffer. To each tube, bovine serum albumin (Fraction V; Fisher Scientific) in 100 μL of borate buffer was added to reduce nonspecific binding at a final concentration of 0.15% for T and 0.19% for E_2. Antibody (Endocrine Sciences) was then added to 200 μL of borate buffer for a final concentration of 1:25,000 for T and 1:55,000 for E_2. Finally, radiolabeled steroid ([2,4,6,7,16,17-3H] E_2 at 1 mCi/mL; [1,2,6,7-3H] T at 1 mCi/mL; Amersham Int., Arlington Heights, IL) was added at 12,000 cpm per 100 μL for a final assay volume of 500 μL. Interassay variance tubes were similarly prepared from two separate plasma pools for E_2 and T. Standards for both E_2 and T were prepared in duplicate at 0, 1.56, 3.13, 6.25, 12.5, 25, 50, 100, 200, 400, and 800 pg/tube. Assay tubes were vortexed for 1 min and incubated overnight at 4°C. Bound-free separation was performed by adding 500 μL of a mixture of 5.0% charcoal to 0.5% dextran, pulse-vortexing, and centrifuging tubes (1500g, 4°C, 30 min). Supernatant (500 μL) was added to 5 mL of scintillation fluid, and counted on a Beckman scintillation counter. Plasma intraassay variance for E_2 and T averaged 2.87% and 4.93%, respectively. Plasma interassay variance for E_2 and T averaged 7.64% and 5.25%, respectively.

Insulin-Like Growth Factor-1 (IGF-1) RIA Biochemical Validation

From each treatment group, plasma (200 μL) was pooled for validation, and was extracted in polypropylene tubes with acid-ethanol (12.5% 2 N HCl, 87.5% ethanol; 800 μL) to dissociate IGF binding proteins from the IGF-I molecules and to precipitate globular proteins as per Daughaday et al. (1980) and Crain et al. (1995). After 30 min incubation (room temperature) and 10-min centrifugation (2500×G; 4°C), the supernatant was aliquoted to produce plasma equivalents of 12.5, 25, 50, 100, and 200 μL. Plasma dilution volumes were brought to 200 μL with acid-ethanol prior to air-drying. Plasma dilutions were compared with 0, 39, 156, 313, 625, 1000, 1250, 2500 pg of human recombinant IGF-1 standard (National Hormone and Pituitary Program, Torrance, CA 90509). Validation samples were examined by IGF RIA procedures as described for experimental sample analyses below. Plasma extraction efficiencies were determined by adding 100 μL iodinated IGF-1 (15,000 cpm) to 100 μL of pooled plasma samples, extracting with acid-ethanol, air-drying, and reading samples on a Beckman 5500B gamma counter to determine the iodinated hormone remaining. The extraction efficiency of plasma was 77.0% and all sample concentrations were corrected for this loss. Validation of plasma dilutions was accomplished in one assay having an intraassay variance of 2.27%.

IGF-1 RIA Procedures

IGF-1 RIA was performed as described by Crain et al. (1995) and Guillette et al. (1994). The National Hormone and Pituitary Program (Torrance, CA 90509) supplied human recombinant IGF-I standard (9.76 to 2500 pg/tube) and human IGF-1 antisera (Lot # AFP4892898, 1:400,000 final dilution). The antiserum had less than 1.0% cross-reactivity with human IGF-II. Iodinated IGF-1 label (IGF-1^{I125} sp act 2000 Ci/mmol; 16,000 cpm/tube) and Amerlex-M donkey anti-rabbit secondary antibody (code RPN510, 500 μL/tube) were supplied through from Amersham International (Arlington Heights, IL). Buffer reagents were purchased from Fisher Chemical Co. (Pittsburgh, PA). Briefly, samples were aliquoted into polypropylene

tubes, extracted with 400 μL acid-ethanol, and incubated 30 min prior to centrifugation (2500×G; 4°C; 10 min). For each sample, supernatant (100 μL) was pipetted into duplicate polypropylene tubes and air-dried. IGF-1 standards were prepared in duplicate with 100 μL of known concentrations of human recombinant IGF-1 standard (ranging from 9 - 2500 pg/tube), and 300 μL RIA buffer (200 mg/L protamine sulfate, 4.14 g/L sodium phosphate monobasic, 0.05% TWEEN 20, 0.02% sodium azide, 3.72 g/L EDTA) added to each tube. Air-dried samples were reconstituted with 350 μL RIA buffer and vortexed. To each sample was added 50 μL IGF-1 antibody (human IGF-1 antisera, UB3-189; 1:10,000 final dilution). After adding 100 μL of iodinated IGF-1 label (I^{125}-IGF-1; 15,000 cpm) samples were vortexed and incubated (4°C) overnight. Separation of bound and free IGF-1 was accomplished by incubating samples for 10 min with 500 μL of secondary (2°) antibody (Amerlex-M donkey anti-rabbit secondary antibody, code RPN.510, Amersham International; 1:10,000 final dilution). Sample tubes were centrifuged (2500×G; 4°C; 10 min) to separate the secondary antibody, which is bound to the primary antibody and ligand. The supernatant was decanted and the pellet counted on a Beckman 5500B gamma counter. Pre-ovariectomy and post-treatment plasma samples were run in two assays having an average intraassay variance of 5.76% and an interassay variance of 4.19%.

Statistics

Wet tissue mass (mg) of liver, oviduct, and ovary were compared among treatment groups using ANCOVA, with body mass as a covariate, followed by LSD post-hoc tests. Data are presented as adjusted means (mg) ± SEM. The oviductal growth parameters were compared among treatment groups with ANOVA followed by Fishers Protected LSD post-hoc test. Log transformation of the data was performed in order to achieve homogeneity of variances prior to ANOVA. Plasma concentrations of E_2, T, and IGF-1 were estimated from raw data using the commercially available Microplate Manager software (Microplate Manager III, BioRad Laboratories, Inc., Hercules, CA, 1988). For RIA validation of pooled plasma dilutions and

internal standards, hormone concentrations were log10-transformed prior to testing for homogeneity of slopes with standard curves by ANCOVA. Plasma concentrations of E_2, T, and IGF-1 were compared among treatment groups with one-way ANOVA followed by Scheffe post hoc. Tamhane post hoc was used where variances were unequal among groups. Statistical analyses were performed using SPSS software (v. 10, SPSS Inc., Chicago, IL, 1999) with $\alpha = 0.05$.

Results

Biochemical RIA Validations

Internal standards and plasma dilutions were parallel to the standard curve for E_2 (ANCOVA; $F = 0.13$, $P = 0.73$ and ANCOVA; $F = 0.01$, $P = 0.91$, Fig. 4-4A), and T RIA (ANCOVA; $F = 0.0001$, $P = 0.99$; Fig. 4-4B). Plasma dilutions were parallel to the standard curve for IGF-1 RIA (ANCOVA; $F = 0.01$, $P = 0.90$; Fig. 4-4C).

Tissue Weights

At the time of the ovariectomy surgeries, excised ovaries contained predominantly mature, highly polarized follicles exhibiting clear demarcation between animal and vegetal hemispheres. Several of the sham females had slightly immature ovaries with mostly yellow and some vitellogenic follicles. Ovary mass did not vary significantly among treatment groups (ANCOVA; $F = 0.16$, $P = 0.16$).

Liver mass was not significantly different among treatment groups (ANCOVA; $F = 0.48$, $P = 0.79$). Oviduct mass was greatest in frogs given E_2 and E_2/IGF-1 compared to those given placebo, EGF, IGF-1, and sham treatments (ANCOVA; $F = 5.14$, $P = 0.001$; Fig. 4-5).

Oviduct Morphometrics

The wall of the oviduct is composed of three distinct morphological regions: the endometrium lined with a lumenal epithelium, the myometrium, and the outer serosa layers. The lumen, or central region of the oviduct, receives secretions synthesized by the epithelial cells and endometrial glands. The epithelial layer forms a continuous boundary surrounding the oviductal

lumen. The endometrial layer, lying internally to the lumenal epithelium, contained secretory glands, connective tissue, and capillaries. The muscle layer, or myometrium, is composed of smooth muscle and forms a continuous external boundary around the endometrium. The outer covering of the oviduct is a relatively thin layer of connective tissue, the serosa. Representative sections from oviducts of frogs under each treatment group are shown (Fig. 4-6, 4-7).

Regardless of treatment group, the oviducts exhibited a ciliated epithelial layer composed primarily of cuboidal cells with darkly staining, basal nuclei. The epithelial cell layer appeared more convoluted in frogs given placebo (Fig. 4-6A), EGF (Fig. 4-6B), IGF-1 (Fig. 4-6C), and sham (Fig. 4-7C) treatments. For frogs given E_2 (Fig. 4-7A) and simultaneous E_2/IGF-1 (Fig. 4-7B) treatment, the epithelial cell layer exhibited little or no convolution, and formed a fairly straight, continuous boundary around the lumen. Epithelial cell height was greater in E_2 and simultaneous E_2/IGF-1 treated frogs compared to controls and other treatment groups (Fig. 4-8A).

Endometrial layer thickness (Fig. 4-8B) and surface area (Fig. 4-8C) were greater for frogs given E_2 and simultaneous E_2/IGF treatments compared to other groups, and no difference in growth as noted between these two treatment groups. The endometrial layer in frogs receiving these treatments contained numerous large and densely arranged glands. Often the gland height extended the entire width of the endometrium. Cells containing abundant cytoplasm, darkly staining nuclei, and a central gland lumen comprised the glands, which also had a duct opening onto the lumenal epithelium. The oviductal glands in frogs receiving placebo, EGF, IGF-1, and sham treatments were much smaller in height, width, and total surface area. The endometrial layer of these frogs was much reduced and connective tissue occupied more relative endometrial space than did the glands.

Plasma Steroid and IGF-1 Concentrations

Pre-ovariectomy plasma hormones were similar in females among treatment groups for E_2 ($P = 0.08$), T ($P = 0.40$), and IGF-1 ($P = 0.30$). Collectively these data indicate that the females were in a similar reproductive stage, and had similar plasma steroid and IGF-1

concentrations before treatments. Thus, their responses to the treatments are unlikely to have been obscured by pre-ovariectomy differences in these parameters.

Plasma E_2 concentrations were decreased significantly after treatment with placebo ($P < 0.001$), EGF ($P = 0.001$), or IGF-1 ($P < 0.001$) but were similar to pre-ovariectomy concentrations for E_2, E_2 /IGF-1, and sham treatment groups (Fig. 4-9). After treatments, plasma E_2 concentrations were greater in E_2, E_2/IGF-1, and sham female compared to placebo, EGF, and IGF-1 treatment groups ($P < 0.001$; Fig. 4-10).

Compared to pre-ovariectomy samples, plasma T concentrations were decreased after placebo ($P = 0.02$), EGF ($P = 0.01$), IGF-1 ($P = 0.002$), E_2 ($P = 0.01$), and E_2/IGF-1 ($P = 0.03$) treatment, but not for sham treatment ($P > 0.05$; Fig. 4-11). Following treatments, plasma T was higher for only the sham group ($P < 0.001$; Fig. 4-12).

Compared to pre-ovariectomy samples, plasma IGF-1 was significantly increased in frogs given IGF-1 ($P = 0.0005$), E_2 ($P < 0.001$), and E_2/IGF-1 (t-test; $P < 0.001$) but lower for placebo, EGF, and sham females (t-test; $P > 0.05$; Fig. 4-13). After treatment, plasma IGF-1 was higher in IGF-1, E_2, and E_2/IGF-1 females than in placebo, EGF, and sham females (ANOVA; $P < 0.001$; Fig. 4-14).

Ovariectomized frogs given placebo, EGF, and IGF-1 exhibited lower post-treatment steroid concentrations compared to pre-ovariectomy concentrations, and verify that the ovariectomy surgeries were successful in removing endogenous ovarian steroid sources. In addition, similar pre-ovariectomy and post-treatment plasma E_2 concentrations for E_2 and E_2/IGF treated frogs, and similar pre-ovariectomy and post-treatment plasma IGF-1 concentrations for IGF and E_2/IGF-1 treated frogs indicate that E_2 and IGF-1 treatments were delivered effectively at physiologically relevant concentrations.

Discussion

Results from this study confirm that E2 stimulates oviductal growth in *R. catesbeiana*. Treatment with growth factors, placebo, and sham produced no oviduct growth. Lastly, treatment

with combined E_2/IGF failed to stimulate a greater oviduct growth than was observed with E_2 treatment alone. In contrast to reptiles and mammals examined using similar technique, *R. catesbeiana* did not exhibit an oviductal growth response with EGF or IGF-1 treatment, nor did they exhibit a synergistic growth response to E_2/IGF-1 treatment. Although E_2 and IGF-1 are not synergistic in stimulation of oviduct growth in *R. catesbeiana*, both hormones might still be required for oviductal growth.

There are several possible explanations for the absence of an oviduct growth response in *R. catesbeiana* to growth factor (or to combined E_2/IGF-1) treatment. First, the IGF-1 treatment doses might have been insufficient to elicit an oviductal growth response in *R. catesbeiana*. Future studies should investigate what doses of IGF-1 are capable of stimulating oviduct growth in ovariectomized *R. catesbeiana*. However, it is unlikely that the IGF-1 dose was insufficient because frogs given IGF-1 exhibited greater post-treatment than pre-ovariectomy plasma IGF-1 concentrations. It is possible that the oviduct must first be "primed" with E_2-stimulation before IGF-1 exposure to become sensitive to the effects of IGF-1. This priming of oviductal tissue might involve E_2-alpha receptors (ERα) and IGF-1 receptors (IGF-1R) upregulation. Klotz et al. (2000) demonstrated that ERα is required for IGF-1 to induce a cellular response. Additionally, Clark et al. (1997) demonstrated that E_2 stimulates proliferative responses of reproductive tissues by upregulating IGF-1R expression, which increases tissue response to circulating IGF-1. These findings imply that an increase in circulatory E_2 concentrations can sensitize receptor-dependent tissue growth IGF-1 stimulation without necessarily requiring an increase in circulating IGF-1 concentrations. As a second explanation, we must consider that circulating steroids can arise from non-gonadal sources such as the adrenal glands (Norris, 1997). As a third explanation, sensitivity to these growth factors represents a relatively recent evolutionary change in reptilian and mammalian oviductal physiology. It is important to recognize that findings reported in this study might be exclusive to *R. catesbeiana*. There are likely interspecific differences in hormonal

regulation of oviduct growth among amphibians. Accordingly, more amphibian species should be examined, using similar techniques, before we can fully understand how amphibian oviduct growth is regulated by interactions of steroids and growth factors.

It is interesting to note that oviduct growth in sham frogs was not similar to growth in E_2- and in E_2/IGF-1 treated frogs. Sham frogs were expected to exhibit oviduct growth, similar to E_2-treated frogs but greater than that of placebo frogs, because their intact ovaries would continue to synthesize and secrete E_2 throughout the study. There are several possible explanations for these unexpected findings. First, it is possible that the implants in E_2- and E_2/IGF-treated frogs contained E_2 concentrations higher than is typically found in *R. catesbeiana*. E_2 doses were determined based on studies of E_2 necessary to elicit oviduct growth in *Xenopus laevis* (Follett and Redshaw, 1967; Redshaw et al., 1968) and in reptiles (Cox, 1994). Thus, these doses might have been comparatively high for *R. catesbeiana*. However, this hypothesis seems unlikely because pre-surgery and post-treatment plasma E_2 concentrations were similar for E_2- and E_2/IGF-1-treated frogs. A second explanation is that sham frogs were different from E_2- and E_2/IGF-1-treated frogs with respect to pre-surgery and post-treatment plasma IGF-1 concentrations. In E_2- and E_2/IGF-1-treated frogs, plasma IGF-1 concentrations increased after treatment compared to pre-ovariectomy levels. In sham frogs, however, post-treatment plasma IGF-1 concentrations did not increase relative to pre-surgery levels. In E_2- and E_2/IGF-1-treated frogs, the increase in plasma IGF-1 could have stimulated increased IGF-1R expression in oviductal tissues, making them more sensitive to E_2- and IGF-1 stimulation. As mentioned previously, IGF-1R does interact, or exhibit "cross-talk" with the ERα in stimulating oviduct growth (Klotz et al., 2002). Since plasma IGF-1 concentrations in sham frogs did not change during the experiment, it is possible that oviductal IGF-1R expression also remained unchanged, making oviductal tissue comparatively less sensitive to E_2- or IGF-1-induced stimulation of growth. Future studies should examine oviduct growth not only in response to steroid and growth factors hormones, but in also in response to changes in ERα and IGF-1R activity to better

understand the role of these receptors in mediating the effects of E_2 and IGF-1 on oviduct growth. Finally, there might have also been an implant effect on oviduct growth. With or without hormones, the implant might have elicited oviductal hypertrophy due to an irritation response of the frogs to implant "foreign body" within the abdomen; this implant effect would have been absent in sham frogs.

Changes in oviductal mass associated with seasonal changes in plasma steroids have been described for a wild population of *R. catesbeiana* (Licht et al., 1984). However, more research is necessary to understand the mechanism by which E_2 induces a growth response in target tissues of amphibians. In mammals, ovarian steroids induced a complex suite of morphological, physiological, and biochemical changes in the oviduct (Buhi et al., 1997). Estrogen-induced oviduct growth relies upon activation of genes that modulate expression of growth factors and their receptors (Murphy and Murphy, 1994; Cox and Guillette, 1994). Estrogen stimulates DNA synthesis and mitosis of epithelial cells, and increases uterine IGF-1 and IGF-1R gene expression. In uterine cells, IGF-1 induces DNA synthesis similar to E_2-stimulation. Thus, activation of the growth factor signaling systems by E_2 is an important part of uterine growth and proliferation in mammals (Klotz et al., 2002; Segars and Driggers, 2002; Driggers and Segars, 2002). It remains unknown whether E_2-induced oviduct growth in amphibians occurs through activation of these growth factors and their receptors, release of IGF-1 from IGF-1 binding proteins, or by another mechanism not yet identified.

No study has comprehensively examined the effects of both growth factors and steroids on oviduct morphology in amphibians. However, both IGF-1 and EGF have been associated with oviduct growth in mammals and reptiles (Cox and Guillette, 1994; Murphy, 1990; DiAugustine et al., 1988). In ovariectomized geckos, IGF-1 and EGF stimulates moderate growth of the oviduct in the absence of E_2 indicating these growth factors play an important role in reptilian reproduction. Also in reptiles, EGF and IGF-1 are known to stimulate oviduct growth directly, although the exact mechanism for proliferation in not known. In amphibians, IGF-1 and IGF-1

binding proteins have been identified in the plasma but the location of IGF-1, IGF-1 binding proteins, and IGF-1 receptors in reproductive tissues of amphibians have not been examined. Location and activity of IGF-1BPs in oviductal tissue, in addition to their interaction with circulating IGF-1 in amphibians, is necessary to understand how these binding proteins regulate IGF-1 activity and oviduct growth.

As expected, plasma IGF-1 concentrations increased with IGF-1 and simultaneous E_2/IGF-1 treatment. However, an unexpected increase in plasma IGF-1 was observed in females treated exclusively with E_2. An interesting endocrine pathway can be described from these findings. The liver is the primary site for synthesis of IGF-1 found in the plasma. Perhaps E_2 stimulated liver IGF-1 synthesis directly, or E_2-stimulated increased pituitary growth hormone release that, in turn, stimulated liver IGF-1 synthesis. Another possible source for the increased plasma IGF-1 in E_2-treated females is the oviduct. There are an increasing number of studies that have identified non-hepatic sources of IGF-1 and examined their role in mediating tissue growth. Numerous studies in reptiles and mammals have shown that the oviduct synthesizes IGF-1 (Cox and Guillette, 1993; Cox, 1994; Le Roith et al., 2001; Driggers and Segars, 2002; Klotz et al., 2002; Segars and Driggers, 2002). As part of separate study not described here, IGF-1 immunoreactivity has been detected in the oviduct of *R. catesbeiana* using immunocytochemistry (T. Barbeau, unpub. obs.). There is some evidence that oviduct-derived IGF-1 affects the oviduct itself in an autocrine manner or affects nearby tissues in a paracrine manner. Whether the oviduct can secrete and contribute significantly to plasma concentrations of IGF-1 remains unknown and is an intriguing area for future research.

In summary, treatment of ovariectomized *R. catesbeiana* with exogenous E_2 and resulted in increased plasma IGF-1 concentrations. This finding indicates that E_2 interacts with the IGF-1 system in amphibians. It remains unknown if increased hepatic or oviductal IGF-1 synthesis and secretion contributed to the increase in plasma IGF-1 concentrations observed in E_2-treated females. The mechanism by which E_2 stimulates increased plasma IGF-1 concentrations in frogs,

and the localization of non-hepatic sources of IGF-1 synthesis in amphibians is poorly understood and requires further investigation.

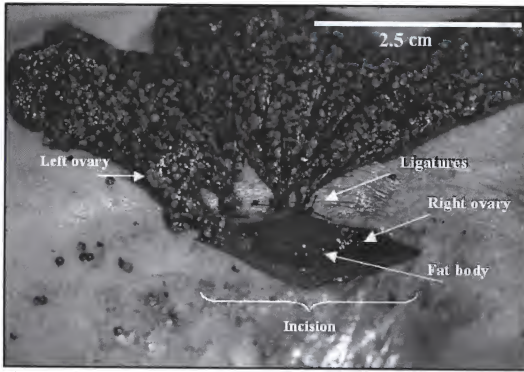

Figure 4-1. Exteriorized right ovary during ovariectomy surgery in *Rana catesbeiana*. Both the left and right ovaries were removed from a 2.5 cm, right paramedial incision into the abdominal cavity.

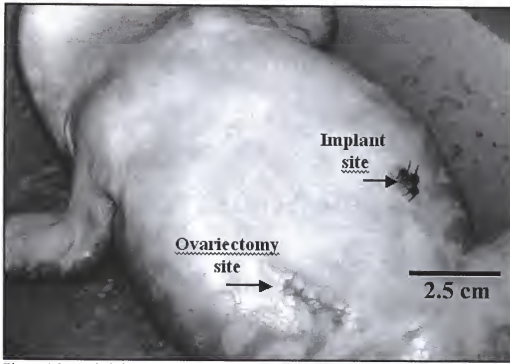

Figure 4-2. Healed right-paramedical incision site visible three weeks after ovariectomy. Also shown is the site of the left paramedial incision in which pellet implants were placed into the abdominal cavity of *Rana catesbeiana*.

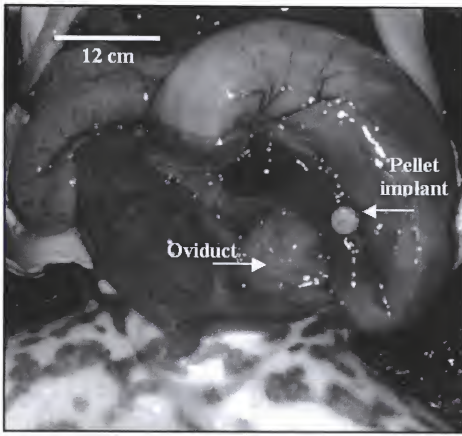

Figure 4-3. Location of intra-abdominal treatment pellet positioned over the left oviduct at time of final dissection, after completion of 18 days of treatment in *Rana catesbeiana*.

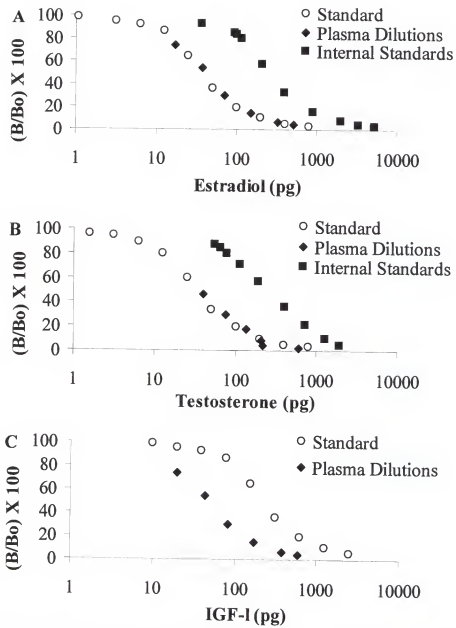

Figure 4-4. Biochemical validation of *Rana catesbeiana* plasma. A. 17β-estradiol RIA internal standards (ANCOVA; F = 0.13; $P = 0.73$) and plasma dilutions (ANCOVA; F = 0.01; $P = 0.91$) were parallel to the standard curve. B. testosterone RIA internal standards (ANCOVA; F = 0.0001; $P = 0.99$) and plasma dilutions (ANCOVA; F = 0.0.08, $P = 0.79$) were parallel to the standard curve. C. insulin-like growth factor-1 (IGF-1) RIA .the plasma dilutions curve was parallel to the standard curve (ANCOVA; F = 0.014; $P = 0.91$).

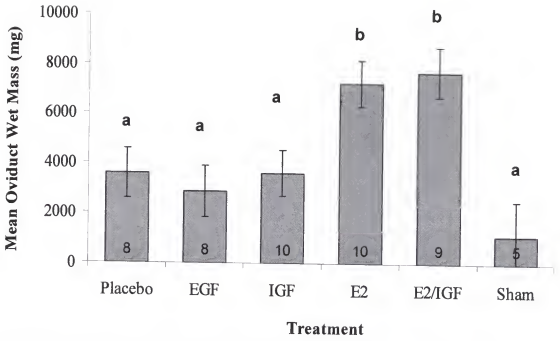

Figure 4-5. Oviduct weights in *Rana catesbeiana* after 18 days of placebo (control), epidermal growth factor (EGF), insulin-like growth factor-1 (IGF-1), 17β-estradiol (E_2), combined E_2/IGF-1, or sham treatment Numbers within bars indicate sample size per treatment while letters above bars indicate significant differences (ANCOVA; $F = 5.14$, $P < 0.001$).

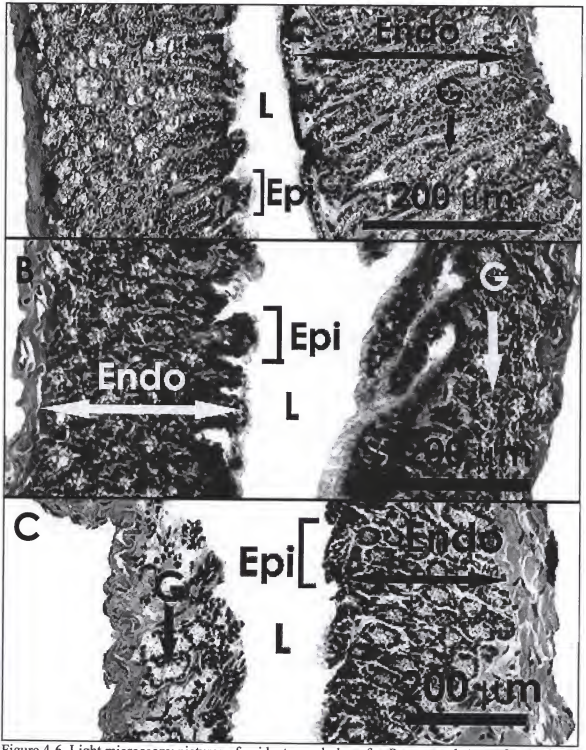

Figure 4-6. Light microscopy pictures of oviduct morphology for *Rana catesbeiana* after 18 days of A. placebo (control), B. epidermal growth factor (EGF), and C. insulin-like growth factor-1 (IGF-1) treatment. Endo = endometrial layer; Epi = epithelium; G = gland; L = lumen.

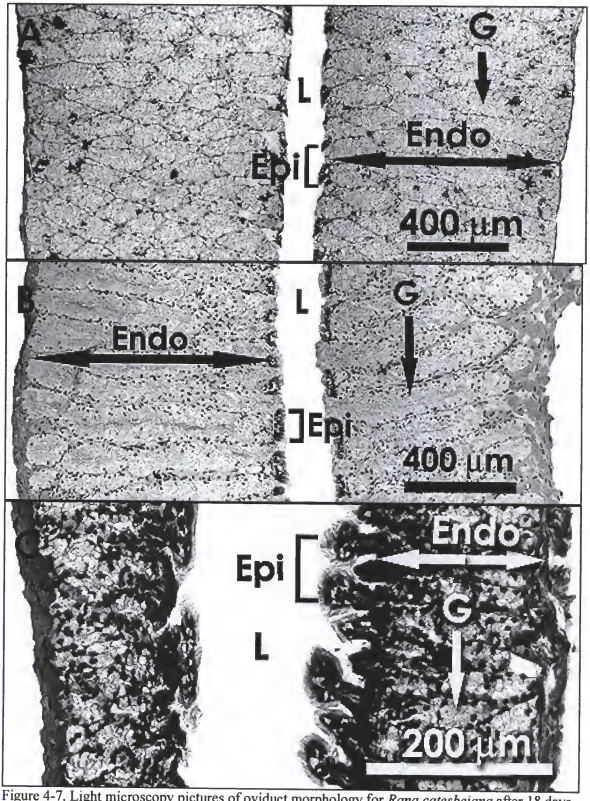

Figure 4-7. Light microscopy pictures of oviduct morphology for *Rana catesbeiana* after 18 days of A. 17-β estradiol (E_2), B. E_2 and insulin-like growth factor-1 (E_2/IGF), and C. sham treatment. Endo = endometrial layer; Epi = epithelium; G = gland; L = lumen.

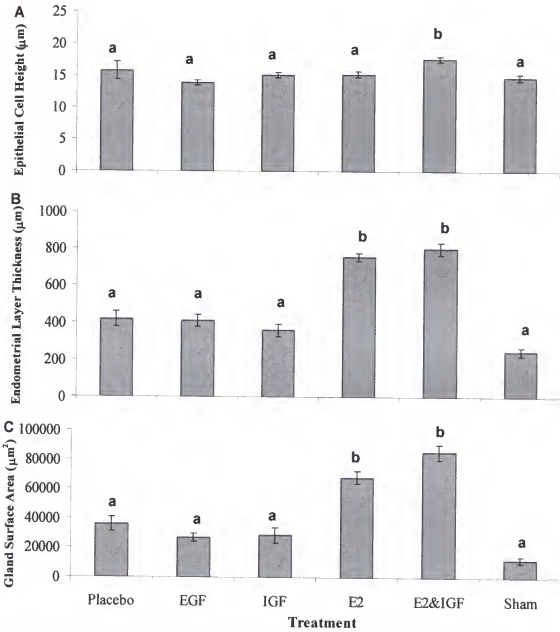

Figure 4-8. Oviduct morphology measurements for *Rana catesbeiana* after 18 days of placebo (control), epidermal growth factor (EGF), insulin-like growth factor-1 (IGF-1), 17β-estradiol (E_2), combined E_2/IGF-1, and sham treatment. Data presented as means ± SEM. Different letters above bars indicate significant differences for A. epithelial cell height (ANOVA; $P < 0.001$), B. endometrial layer thickness ((ANOVA; $P < 0.001$), and C. gland surface area (ANOVA; $P < 0.001$).

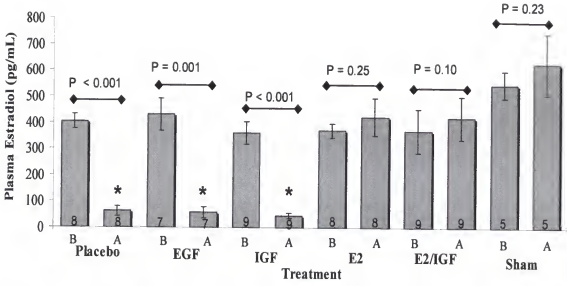

Figure 4-9. Comparison of plasma 17β-estradiol (E_2) concentrations for *Rana catesbeiana* before and after 18 days of placebo (control), epidermal growth factor (EGF), insulin-like growth factor-1 (IGF-1), E_2, combined E_2/IGF-1, or sham treatment. Data presented as means ± SEM. Numbers within bars indicate sample size per treatment. Parallel lines above bars, and letters below bars indicate before (B) and after (A) contrasted pairs. Asterisks and P-values above bars indicate significant differences as determined by paired t-tests.

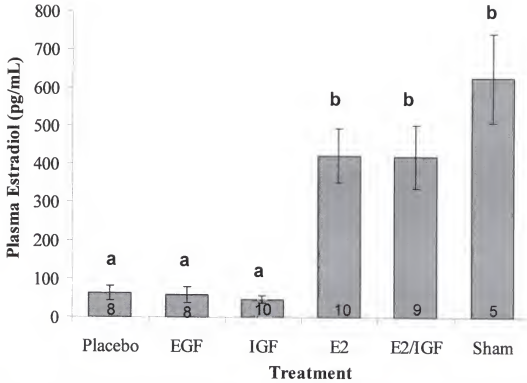

Figure 4-10. Plasma 17β-estradiol (E_2) concentrations for *Rana catesbeiana* after 18 days of placebo (control), epidermal growth factor (EGF), insulin-like growth factor-1 (IGF-1), E_2, combined E_2/IGF-1, or sham treatment. Data presented as means ± SEM. Numbers within bars indicate sample size per treatment. Different letters above bars indicate significant differences among treatment groups (ANOVA; $P < 0.001$).

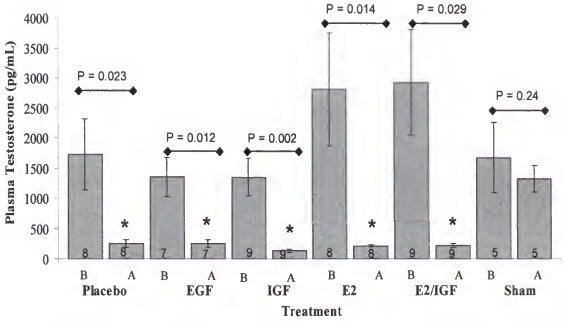

Figure 4-11. Comparison of plasma testosterone concentrations for *Rana catesbeiana* before and after 18 days of placebo (control), epidermal growth factor (EGF), insulin-like growth factor-1 (IGF-1), 17β-estradiol (E_2), combined E_2/IGF-1, or sham treatment. Data presented as means ± SEM. Numbers within bars indicate sample size per treatment. Parallel lines above bars and letters below bars indicate before (B) and after (A) contrasted pairs. Asterisks and P-values above bars indicate significant differences as determined by paired t-tests.

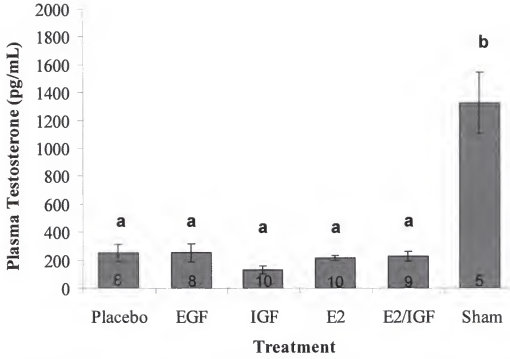

Figure 4-12. Plasma testosterone concentrations for *Rana catesbeiana* after 18 days of placebo (control), epidermal growth factor (EGF), IGF-1, 17β-estradiol (E_2), combined E_2/IGF-1, or sham treatment. Data presented as means ± SEM. Numbers within bars indicate sample size per treatment. Different letters above bars indicate significant differences among treatment groups (ANOVA; $P < 0.001$).

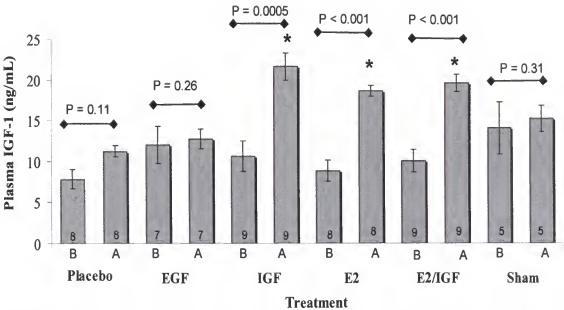

Figure 4-13. Comparison of plasma insulin-like growth factor-1 (IGF-1) concentrations for *Rana catesbeiana* before and after 18 days of placebo (control), epidermal growth factor (EGF), IGF-1, 17β-estradiol (E_2), combined E_2/IGF-1, or sham treatment. Data presented as means ± SEM. Numbers within bars indicate sample size per treatment. Parallel lines above bars, and letters below bars indicate before (B) and after (A) contrasted pairs. Asterisks and P-values above bars indicate significant differences as determined by paired t-tests.

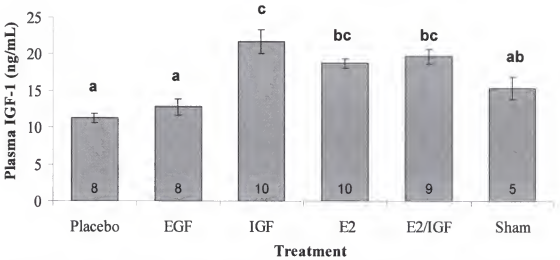

Figure 4-14. Plasma insulin-like growth factor-1 (IGF-1) concentrations for *Rana catesbeiana* after 18 days of placebo (control), epidermal growth factor (EGF), IGF-1, 17β-estradiol (E_2), combined E_2/IGF-1, or sham treatment. Data presented as means ± SEM. Numbers within bars indicate sample size per treatment while letters above bars indicate significant differences among treatment groups (ANOVA; $P < 0.001$).

CHAPTER 5
OVARIAN STEROIDOGENESIS (*IN VITRO*) IN PIG FROGS (*Rana grylio*) AFTER EXPOSURE TO ENVIRONMENTALLY RELEVANT CONCENTRATIONS OF NITRATE AND NITRITE

Introduction

In recent decades, dramatic declines of amphibian populations have been documented worldwide. Exposure to man-made environmental contaminants is considered an important contributor to these declines (Blaustein and Wake, 1990; Carey and Bryant, 1995; Hayes et al., 2002). Recently, nitrate contamination of watersheds has received increasing scientific scrutiny. Studies indicate that nitrogenous fertilizers contribute to the decline of some amphibian populations within agricultural landscapes (Berger, 1989; Hecnar, 1995; Baker, 1993; Oldham et al., 1997; Marco et al., 1999). Unfortunately, more research is needed to understand the harmful effects of nitrate contamination on amphibian populations.

Nitrate is a natural part of biochemical cycling within terrestrial and aquatic ecosystems. Nitrate is an anionic form of nitrogen produced by bacterial nitrification. Atmospheric nitrogen (N_2) is fixed by symbiotic bacteria within the root nodules of legumous plants, and by free-living bacteria in soil and water into ammonia. Ammonia is then converted by nitrifying bacteria (*Nitrosomonas* sp.) into nitrite and by *Nitrobacter* sp. into nitrate (Painter, 1975). Nitrate is the most stable ionic form of nitrogen that can be assimilated by plants or converted back into nitrite and nitrogen by denitrifying bacteria. Excessive amounts of nitrite and nitrate can overwhelm the recycling capacity of nitrifying and denitrifying bacteria, resulting in elevated concentrations of nitrite and nitrate within the environment.

Sources of excessive nitrogen input into the environment include runoff of nitrogenous fertilizers, runoff of industrial effluent, leakage of human and animal wastes, and atmospheric

pollution. Each year, over 10 million tons of nitrogenous fertilizers are used in the US, and over 72 million tons are used globally (International Fertilizer Industry Association, 1993). In the United States, use of nitrogenous fertilizers increased 20-fold between 1945-1993 (Pucket, 1995) and currently exceeds 10 million tons. The application of fertilizers in close proximity to watersheds during the spring often overlaps the breeding season of many amphibians. An estimated 10-60 % of the nitrogen from fertilizer application remains unused by crops and leaches into groundwater or washes into surface water as nitrate with rainfall (Ministry of Agriculture, Fisheries, and Food, 1993). Animal manure contributed approximately 6 million tons of nitrogen input to the environment. Leakage of human wastes from septic tanks and poorly maintained sewage systems contributed a small percentage to nitrogen input. Finally, 3 million tons of nitrogen from atmospheric sources including burning of fossil fuels, automobile exhaust, and industrial emissions contributed to soil and water nitrogen input (Pucket, 1995). Nitrogen from these sources is carried by rainfall into surface waters, leached from the soil into ground water, and deposited into major subterranean aquifers. Since nitrate values are commonly reported in the literature as nitrate in nitrogen (NO_3-N), for the remainder of this chapter nitrate refers to NO_3-N (Chapter 1, Table 1-1).

In streams and lakes of North American, nitrate concentrations range from 2 and 40 mg/L and can persist for long time periods (Rouse et al., 1999). Globally, concentrations of nitrate in aquatic ecosystems surrounding agricultural and urban landscapes can exceed 100 mg/L (Chilton, and Foster, 1991; Fried, 1991). The US Geological Survey National Water-Quality Assessment (NAWQA) Program conducted a 3-year study of 50 prominent hydrologic systems within the United States to determine the concentration of nitrates in groundwater. Nitrate was detected in 71% of the groundwater sites examined. Shallow groundwater beneath agricultural land had median nitrate concentrations of 3.4 mg/L nitrate. Of 21 sites where nitrate was examined, 13 sites exceeded the US EPA maximum contaminant limit (MCL) of 10 mg/L nitrate (Nolan and Stoner, 1995).

Nitrate contamination in aquatic ecosystems and in drinking water poses serious health consequences for wildlife and humans. Nitrate and nitrite enter the body by consumption of food and water, by crossing the gill epithelia in aquatic organisms (*e.g.*, fishes and tadpoles), and by absorption through the skin. Nitrate and nitrite are transported against concentration gradients by substituting for chloride in the bicarbonate-chloride exchange in normal osmoregulatory and respiratory functions (Lee and Pritchard, 1985; Doblander and Lackner, 1996; Jensen, 1996; Evans, 1999; Panesar, 1999). These anions can accumulate within extracellular fluid and tissues. Bacteria within the mouth and gastrointestinal tract convert nitrate into nitrite and N-nitrosamines. Nitrites are highly toxic and induce methhemoglobinemia, especially in children exposed to concentrations in drinking water greater than 40 mg/L (US EPA, 1995). Nitrite oxidizes the iron within the heme subunits of hemoglobin converting it to methhemoglobin. Methhemoglobin has a reduced ability to transport oxygen and results in cyanosis. For this reason, the phrase "blue-baby syndrome" is used to describe the condition of infants that develop methhemoglobinemia from drinking formula made with nitrate-contaminated water (US EPA, 1995). In over 40 animal species examined, including primates, N-nitrosamines have been shown to be carcinogenic (Weyer et al., 1986). For these reasons, the United States Environmental Protection Agency (US EPA) has recommended a strict MCL of 10 mg/L for nitrate and 1.0 mg/L for nitrite in drinking water to (US EPA, 1995). The recommended limit for nitrate in aquatic ecosystems is 90 mg/L nitrate for freshwater fishes and is based on a lethal exposure values. Unfortunately these limits are often exceeded due to human activity in agricultural and urban areas.

Most studies that report the effects of nitrate on amphibians have focused on lethal concentrations that induce easily observed physiological toxic responses resulting in mortality. Few studies have examined the effects of exposure to sublethal concentrations of nitrate on amphibians (Rouse et al., 1999). Sublethal exposure to contaminants can disrupt the endocrine system in amphibians resulting in altered reproduction, abnormal growth, or deformities (Cooke,

1981; Mohanty-Hejmadi and Dutta, 1981; Reeder et al., 1998; Kloas et al., 1999). Studies that report sublethal effects of nitrate exposure on amphibians have examined only tadpole life-stages (Table 1-2). Exposure to nitrate concentrations ranging from 2.5 - 100 mg/L nitrate induced sublethal effects in tadpoles such as reduced feeding activity, nostral and tail deformities, and erratic swimming behavior. For some species, exposure of tadpoles to nitrate concentrations as low as 0.78 - 6.0 mg/L nitrite is lethal after 15 days of exposure (Chapter 1, Table 1-2). Variable responses of amphibians to lethal and sublethal concentrations indicate species differences in susceptibility to perturbation by nitrate, and effects vary according to life-stages under which exposure occurs (Marco et al, 1999; De Solla et al., 2002).

Recent research has shown that exposure to relatively high concentrations of nitrate and nitrite inhibits synthesis (*in vivo* and *in vitro*) of testosterone (T) in rodents (Panesar, 1999; Panesar and Chan, 2000). Mature bulls consuming nitrate-contaminated feed for 30 days also exhibited decreased plasma T concentrations (Zraly et al., 1997). In alligators, *in vivo* exposure to low nitrate concentrations (1.65 mg/L nitrite) increased plasma steroid concentrations but *in vitro* exposure of testes to nitrate (1.65 mg/L nitrate) results in decreased T synthesis (Guillette and Gunderson, unpubl. data). This variable pattern of endocrine disruption associated with *in vitro* versus *in vivo* nitrate exposure indicates that the effects of nitrate depend on route of exposure. Despite findings of nitrate-induced steroid hormone inhibition in these animals, no study has examined whether nitrite and nitrate act as endocrine-disruptors in amphibians.

In this study, I examined wild-caught Pig frogs (*Rana grylio*) for steroid hormones, 17β-estradiol (E_2) and T, synthesized by the ovaries (*in vitro*) in response to nitrate and nitrite exposure at environmentally relevant concentrations. Based on the studies already described, I predicted that nitrate and nitrite inhibits synthesis of steroid hormones.

Materials and Methods

Collection of Animals

All work was conducted under the approval of the University of Florida Institute of Animal Care and Use Committee (IACUC project #Z023). During the breeding season of 2003, a total of 10 female *R. grylio* were collected from Orange Lake, Alachua County, Florida. Five females were collected separately in both June and July. Frogs were transported to the Department of Zoology and anesthetized, and necropsied, within 12 h of capture. The ovaries were harvested, weighed, and individual ovarian follicles were dissected for an *in vitro* culture study. Ovarian follicles of specific maturation stages 4, 5, and 6 were selected for culture because they synthesize E_2 and T (Fortune, 1981).

Ovarian Follicle Culture *In Vitro*

Preliminary *in vitro* ovarian follicle cultures demonstrated that 3 h of culture was the optimum time for measuring steroid concentrations synthesized by *R. grylio* ovarian follicles (P = 0.007, Figure 5-1). From each frog, 33 follicles each of stages 4, 5, and 6 were incubated under ambient air conditions in 35×10 mm sterile culture dishes at 29ºC with 3 mL of sterile, phenol-free culture media (1L M199 HBSS, 3.4 mL 200 mM L-glutamine, 5.96 g/L HEPES, 0.35 g/L sodium bicarbonate, 8.0 mL 0.1 mM IBMX, pH 6.9; Sigma-Aldrich, St. Louis, MO). For each of the 10 females, 99 follicles were cultured for 3 h under each of the following treatment groups: no nitrate or nitrite (control), 0.17, 1.65, 6.60, 16.50, and 33.00 mg/L nitrate, and 0.20, 2.03, 8.12, 20.30, and 40.60 mg/L nitrite for a total of 11 treatment groups.

The control treatment group represents steroid synthesis in the absence of nitrate and nitrite whereas the remaining treatments represent follicular steroid synthesis in the presence of varying concentrations of nitrate or nitrite. After 3 h of incubation, culture media was decanted, flash-frozen, and stored at -70ºC for RIA analyses of E_2 and T concentrations among treatment groups.

Steroid Radioimmunoassay (RIA) Procedures and Validations

RIAs were performed for T and E_2 on culture media using validated procedures. Duplicate media samples (50 μL for T and E_2) were twice extracted with ethyl-ether, air-dried, and reconstituted in borate buffer (0.05 M; pH 8.0). Antibody (Endocrine Sciences) was added at a final concentration of 1:25,000 for T and 1:55,000 for E_2. Radiolabeled steroid ([2,4,6,7,16,17-^{3}H] estradiol at 1 mCi/mL; [1,2,6,7-^{3}H] and testosterone at 1 mCi/mL; Amersham Int., Arlington Heights, IL) was added at 12,000 cpm per 100 μL for a final assay volume of 500 μL. Interassay variance tubes were prepared from two separate pools of media and plasma for T and E_2. Standards for T and E_2 were prepared in duplicate at 0, 1.56, 3.13, 6.25, 12.5, 25, 50, 100, 200, 400, and 800 pg/tube. Assay tubes were vortexed and incubated overnight at 4°C.

Bound-free separation was performed adding to each tube 500 μL of solution of 5.0% charcoal to 0.5% dextran, pulse-vortexing, and centrifuging tubes (1500g, 4°C, 30 min). Supernatant was added to 5.0 mL of scintillation cocktail, and counted on a Beckman scintillation counter. Media RIA intraassay and interassay variance for E_2 averaged 3.31% and 0.987%, respectively while for T averaged 3.12% and 1.94%, respectively.

Validation of the steroid assay was accomplished with media dilutions (25, 50, 100, 200, and 300 μL for E_2 and 6.25, 25, 50, 100, and 200 μL for T) and with internal standards created by spiking stripped media samples with E_2 and T standards (1.06, 3.125, 6.25, 12.5, 25, 50, 100, 200, 400, and 800 pg per tube). Internal standard and media dilution curves were compared to E_2 and T standard curves to verify homogeneity of slopes. Validation RIA intraassay and interassay variance for E_2 averaged 3.51% and 4.67%, respectively. Validation RIA intrassay and interassay variance for T averaged 3.00% and 5.58%, respectively.

Internal standards and media dilutions for E_2 were parallel to the standard curve ($F = 0.36$, $P = 0.56$ and $F = 0.002$, $P = 0.97$; Fig. 5-2A). Additionally, internal standards and media dilutions for T were parallel to the standard curve ($F = 0.009$, $P = 0.93$ and $F = 0.15$, $P = 0.71$;

Fig. 5-2B). Average recovery of E_2 was 92.2% and of T was 88.0% after media extractions. Final media hormone concentrations were compensated to reflect these losses.

Statistics

Concentrations of T and E_2 in cultures were estimated from raw data using Microplate Manager software (Microplate Manager III, BioRad Laboratories, Inc., Hercules, CA, 1988). Because separate groups of females were collected in June and July, unpaired t-tests were used to compare hormone concentrations between months, for each treatment group, to verify steroid synthesis was similar for females collected during both months. Hormone concentrations were compared among treatments using one-way repeat measures ANOVA followed by Fishers Protected LSD post-hoc. For RIA validation of pooled media dilutions and internal standards (T and E_2), hormone concentrations were log10-transformed prior to testing for homogeneity of slopes with standard curves by ANCOVA. Statistical analyses were performed using SPSS software (v. 10, SPSS Inc., Chicago, IL, 1999) with $\alpha = 0.05$.

Results

Analyses revealed similar patterns in the hormonal responses among the treatment groups for E_2 and T (Fig. 5-3). No significant differences in hormone concentrations, per treatment group, or between months were detected (Table 5-1). Therefore, for comparisons of *in vitro* ovarian steroid synthesis among treatment groups, analyses were conducted with a final sample size of 10 females per treatment group.

After 3 h culture, ovarian E_2 synthesis was significantly reduced at all concentrations of nitrate and nitrite (ANOVA; $P < 0.001$, Figure 5-4). A similar pattern was observed of reduced ovarian T synthesis was observed among treatment groups (ANOVA; $P < 0.001$, Figure 5-5). No clear dose-response pattern in steroid synthesis was observed and hormone synthesis was similarly inhibited by all nitrate and nitrate concentrations.

Discussion

This study provides the first report of nitrate- and nitrite-induced endocrine disruption in the cultured ovaries of adult, wild-caught frogs. Perhaps most concerning is the finding of decreased *in vitro* ovarian steroidogenesis after exposure to sublethal, environmentally relevant concentrations of nitrate and nitrite; the concentrations used here are commonly detected in the surface and groundwater of the United States, and also in human drinking water. Decreased ovarian steroidogenesis (*in vitro*) in *R. grylio* was similar to that reported for *X. laevis* females exposed to nitrate (*in vivo*) at comparatively higher concentrations (Chapter 2).

Ovarian steroid synthesis did not exhibit a linear decrease in concentrations, a typical dose-response curve, with exposure to increasing concentrations of nitrate and nitrite. At 0.17 mg/L nitrate and 0.2 mg/L nitrite, steroid synthesis was already clearly reduced compared to control treatment but similar to other nitrate and nitrite treatments. This finding indicates that even lower concentrations of nitrate and nitrite should be examined in future studies. Lower concentrations might help determine the point at which nitrate and nitrite exposure begins to inhibit steroid synthesis (*in vitro*). Conversely, exposure of ovarian follicles to concentrations as high as 100 or 200 mg/L nitrate and nitrite might demonstrate the point at which steroid synthesis ceases entirely.

In mammals and reptiles, nitrate and nitrate alter steroidogenesis both *in vivo* and *in vitro*. Panesar and Chan (2000) reported that male rodents given nitrate in drinking water (50 mg/L) exhibited a marked reduction in plasma T. *In vitro* culture of mouse Leydig tumor cells with nitrate and nitrate (1000 mg/L) similarly decreased T synthesis. Adult bulls treated with nitrate in their feed exhibited decreased plasma T concentrations after 30 days of exposure. In male alligators, culture of testes with 1.65 mg/L nitrate is associated with decreased T synthesis (*in vitro*). However, nitrate exposure of alligators (*in vivo*), via an i.v. injection, results in increased plasma steroid concentrations indicating that steroid responsiveness is dependent on route of exposure (Guillette and Gunderson, unpubl. data). In amphibians, both adult female *X. laevis* and

R. grylio ovarian steroid synthesis is decreased with nitrate exposure. It is unknown if *in vivo* exposure of *R. grylio* results in decreased ovarian synthesis and plasma steroid concentrations similar to *in vitro* findings reported here. However, the difficulty in maintaining *R. grylio* in captivity for extended time periods makes this kind of comparative study difficult.

A mechanism by which nitrate and nitrite interferes with steroid synthesis has been proposed for mammals by Panesar and Chan (2000). This mechanism has been modified to reflect similar pathways of steroid inhibition described for the ovarian follicle of *X. laevis* (Chapter 2). Briefly, nitrate reacts with sulfhhydryl groups to create nitric oxide (NO) in most body tissues (Mayer and Hemmenns, 1997; Panesar and Chan, 2000; Iniguez et al., 2001). Nitric oxide is a biochemically reactive gas that easily diffuses into tissues and can induce a variety of cellular responses including vasodilation, smooth muscle relaxation, and steroid regulation (McDonald and Murad, 1995; Lincolm et al., 1995; Panesar and Chan, 2000; Bastian et al., 2002). Panesar and Chan (2000) proposed that nitrate either consumed or absorbed into the body is converted into nitrite by bacteria within the mouth and gastrointestinal tract. Nitrite diffuses into cells and is converted into NO by activity of nitric oxide synthase (NOS) enzymes found within most body tissues (Forstermann et al., 1995; Szabo and Thiemermann, 1995; Wang and Marsden, 1995). Both nitrite and NO can interfere with the iron containing proteins found in hemoglobin and in heme subunits of cytochrome P450 enzymes (Jensen, 2003). Free cholesterol is imported, by steroid acute regulatory protein (StAR protein), into the endoplasmic reticulum and mitochondria of steroidogenic tissue, where cholesterol is converted into progesterone (P_4) by P450-side-chain cleavage ($P450_{scc}$) and 3β-hydroxysteroid dehydrogenase (3β-HSD) enzymes. From P_4, T is synthesized and then can aromatized into E_2 (Chapter 2, Figure 2-7). Research indicates that NO interferes with or blocks the activity of StAR, 3β-HSD, and P450 enzymes, thereby inhibiting steroid synthesis at multiple points in the synthetic pathway. Indeed, NO has been shown to decrease synthesis of E_2 and expression of aromatase enzymes in the ovarian

granulosa cells of humans, rodents, cows, and pigs (VanVoorhis et al., 1994; Olson et al., 1996; Srivastava et al., 1997; Basini et al., 1998). Thus, nitrate and nitrite have been shown to alter steroidogenesis in mammals, reptiles, and now in amphibians. The common mechanism for steroid inhibition in these animals likely involves NO interference of steroidogenic enzymes and proteins within the gonad.

The present study examined only *in vitro* gonadal E_2 and T synthesis. Progesterone, NOS, NR, and NO were not analyzed in the culture media or gonadal tissues. Elevated levels of NOS, NR, and NO in culture media and tissues exposed to nitrate and nitrite compared to control samples would indicate increased synthesis or activity of these NO-generating agents. For example, it would be valuable in future studies to examine how NO agonists and inhibitors, given in conjunction with nitrate and nitrite treatment, affect ovarian steroid synthesis (*in vitro*). If steroid synthesis in the presence of nitrate and nitrite remained elevated with the addition of NO inhibitors, yet is suppressed in the presence of NO donors, this would imply that NO activity is the causal agent in steroid inhibition.

More research is necessary to evaluate the long-term reproductive impacts of exposure of amphibians and other vertebrates to nitrate and nitrite. There is already considerable evidence that nitrate and nitrite exposure decreases reproductive hormone concentrations in a variety of animals. The discovery of nitrate- and nitrite-induced steroid inhibition in frogs is concerning because it occurs at concentrations deemed acceptable for human drinking water by current US EPA standards. Impaired reproductive tissue function must now be considered a potential threat to reproduction for wild frogs exposed to low concentrations of environmental nitrate and nitrite. Further studies are needed to determine whether disruption of reproductive hormones by long-term exposure to nitrate and nitrite is a prominent factor in the declines in populations of wild frogs.

Table 5-1. Comparisons of *in vitro* ovarian testosterone and 17β-estradiol concentrations in culture media with *Rana grylio* ovarian follicles incubated for 3 h in the absence (0 mg/L) or presence of nitrate and nitrite. For each treatment group, steroid concentrations were compared between June and July females using T-tests. Steroid concentrations in table presented as means ± SEM. Significance values in table show no significant ($P > 0.05$) differences between June and July means for each treatment group. (SPSS V. 10.1 ($\alpha = 0.05$).

		June (N = 5)	July (N = 5)	
Hormone	**Treatment**	**Mean ± SEM**	**Mean ± SEM**	***P-value***
Testosterone (pg/mL)	0 mg/L	1011.5 ± 67	783.9 ± 68	0.25
	0.172 mg/L nitrate	451.8 ± 56	369.1 ± 32	0.24
	1.65 mg/L nitrate	483.0 ± 100	478.1 ± 117	0.98
	6.60 mg/L nitrate	408.4 ± 26	364.1 ± 21	0.24
	16.50 mg/L nitrate	436.5 ± 79	387.1 ± 59	0.62
	33.00 mg/L nitrate	466.9 ± 49	399.0 ± 72	0.46
	0.20 mg/L nitrite	388.61 ± 62	421.2 ± 61	0.72
	2.03 mg/L nitrite	455.0 ± 81	470.3 ± 73	0.89
	8.12 mg/L nitrite	414.8 ± 52	397.7 ± 43	0.81
	20.30 mg/L nitrite	388.8 ± 71	515.7 ± 104	0.35
	40.60 mg/L nitrite	404.2 ± 65	542.9 ± 127	0.36
Estradiol (pg/mL)	0 mg/Lm	165.5 ± 19	200.2 ±19	0.15
	0.172 mg/L nitrate	135.3 ± 9	118.1 ± 14	0.59
	1.65 mg/L nitrate	150.1 ± 20	99.9 ± 20	0.13
	6.60 mg/L nitrate	148.0 ± 17	93.8 ± 17	0.74
	16.50 mg/L nitrate	103.9 ± 12	91.1 ± 12	0.52
	33.00 mg/L nitrate	121.1 ± 18	108.1 ± 18	0.44
	0.20 mg/L nitrite	119.1 ± 21	111.8 ± 21	0.81
	2.03 mg/L nitrite	120.5 ± 17	79.9 ± 19	0.08
	8.12 mg/L nitrite	117.3 ± 15	116.7 ± 15	0.99
	20.30 mg/L nitrite	104.5 ± 13	91.0 ± 13	0.44
	40.60 mg/L nitrite	106.4 ± 15	92.2 ± 15	0.40

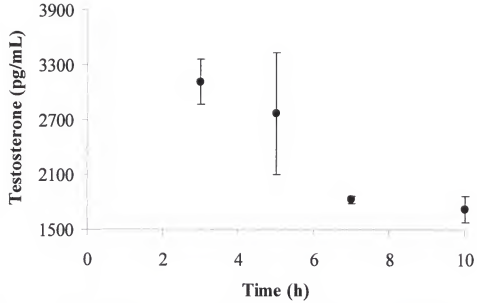

Figure 5-1. Time trial for *in vitro* testosterone concentrations synthesized from female *Rana grylio* ovarian follicles cultured for time intervals of 3, 5, 7, and 10 h. Data presented as means ± SEM. Numbers within axis indicate sample sizes and different letters above the bars indicate significantly different means (ANOVA; $P = 0.007$).

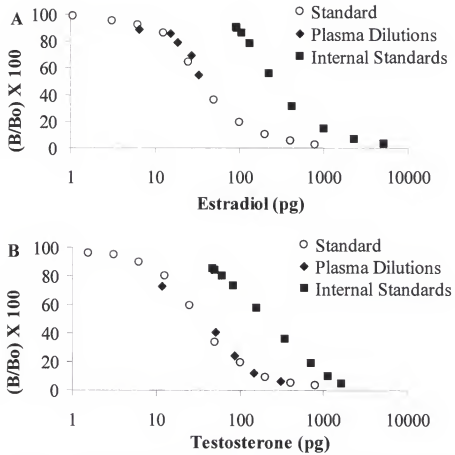

Figure 5-2. Biochemical validation of media from *Rana grylio* ovarian follicle culture. A For 17β-estradiol RIA internal standards and plasma dilution curves were parallel to the estradiol standard curve (ANCOVA; F = 0.36, $P = 0.56$ and F = 0.0.002, $P = 0.97$). B. For testosterone RIA internal standards and plasma dilution curves were parallel to the testosterone standard curve (ANCOVA; F = 0.009, $P = 0.93$ and F = 0.05, $P = 0.71$).

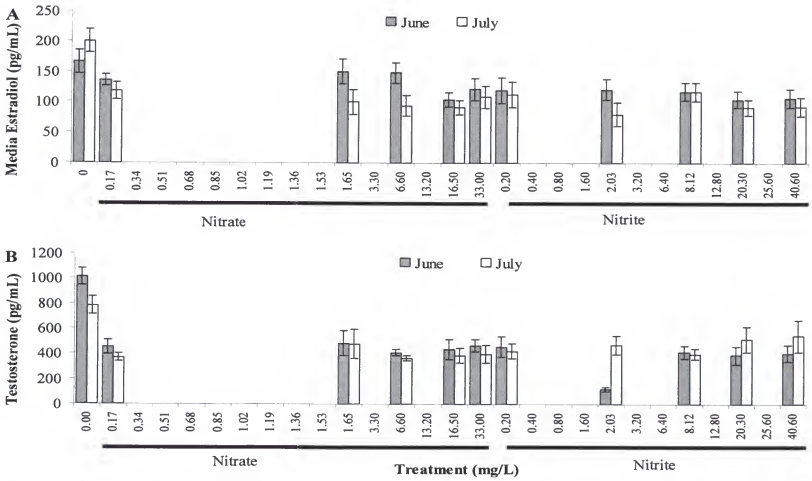

Figure 5-3. A. *In vitro* 17β-estradiol and B. testosterone concentrations in culture media from *Rana grylio* ovarian follicles incubated for 3 h in the absence (O mg/L) or presence of nitrate and nitrite. Data from female *R. grylio* collected during June and July are presented as means ± SEM.

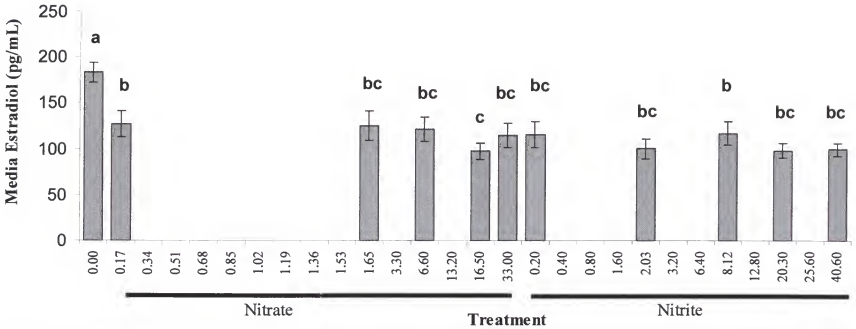

Figure 5-4. *In vitro* 17β-estradiol concentrations in culture media from *Rana grylio* ovarian follicles incubated for 3 h in the absence (O mg/L) or presence of nitrate and nitrite. Data presented as means ± SEM. Numbers within bars indicate sample sizes and different letters above bars indicate significantly different means (ANOVA; $P < 0.001$).

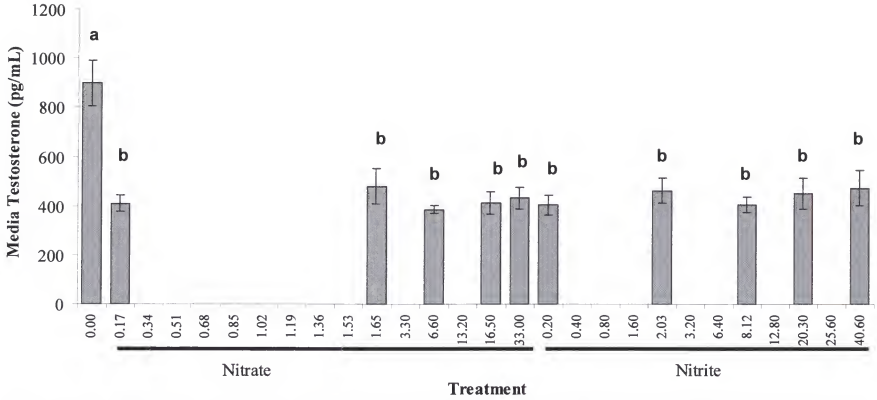

Figure 5-5. *In vitro* testosterone concentrations in culture media from *Rana grylio* ovarian follicles incubated for 3 h in the absence (O mg/L) or presence of nitrate and nitrite. Data presented as means ± SEM. Numbers within bars indicate sample sizes and different letters above bars indicate significantly different means (ANOVA; $P < 0.001$).

CHAPTER 6
THE EFFECTS OF EXPOSURE TO ENVIRONMENTALLY RELEVANT CONCENTRATIONS ON NITRATE ON OVIDUCTAL MORPHOLOGY AND PLASMA STEROIDS AND INSULIN-LIKE GROWTH FACTOR-1 IN BULLFROGS (*Rana catesbeiana*)

Introduction

Nitrate is a widespread contaminant of surface and ground waters worldwide (Halberg, 1989; Nolan and Stoner, 1995; Pucket, 1995). Nitrate is an anionic form of nitrogen that is produced in soil and water by bacterial nitrification of nitrogen gas, ammonia, and nitrite within soil and water (Painter, 1975). Of these forms of nitrogen, nitrate is the most water-soluble and usable by plants. Excessive quantities of nitrate within soil and water often remain largely unused by plants resulting in accumulation in the environment. Although nitrate is less toxic than ammonia and nitrite, it is relatively stable in the environment and can persist in high concentrations for decades (Nolan and Stoner, 1995). Nitrate concentrations in ground and surface waters of the United States have been rising for the past 20 years (Nolan and Stoner, 1995). Nitrates infiltrate ground and surface waters primarily from runoff of inorganic fertilizer, runoff of animal wastes from pastures and feedlots, and leakage of improperly contained human sewage (Nolan and Stoner, 1995; Berndt et al., 1998). Nitrate values are commonly reported in the literature as nitrate-as-nitrogen (NO_3-N), and this is how nitrate is reported for the remainder of this chapter.

Nitrate contamination poses serious health consequences for wildlife and humans alike. When nitrate enters the body, either through consumption or absorption across epithelial surfaces, bacteria within the mouth and gastrointestinal tract convert it into harmful nitrite and N-nitrosamines. Nitrite is highly toxic and induces methhemoglobinemia while N-nitrosoamines are known to cause cancer in over 40 animal species examined. Based on these studies, the US EPA established a strict maximum contaminant limit (MCL) for nitrate in human drinking water of 10

mg/L nitrate. However, wells examined in and around agricultural landscapes frequently exceed this concentration (Nolan and Stoner, 1995). For example, people drinking water from nitrate-contaminated wells exhibit increased incidence of bladder, brain, and colon cancer, and also Non-Hodgkin's lymphoma (Ward et al., 1996, 2000; Weyer et al., 2000; Gulis et al., 2002).

In recent decades, researchers have been concerned over the deformities and the apparent declines in populations of amphibians worldwide (McCoy, 1994; Pounds and Crump, 1994; Laurance et al., 1996; Licht, 1995). Exposure of amphibians to man-made environmental contaminants is considered a prominent contributor to incidents of declines and deformities (Carey and Bryant, 1995; Carey et al., 1999; Hayes et al., 2000). Exposure to contaminants disrupts the endocrine system in amphibians resulting in altered reproduction, abnormal growth, or deformities (Chapter 1, Table 5-1). Research indicates that nitrogenous fertilizers contribute to the decline of some amphibian populations located in agricultural lands (Berger, 1989; Hecnar, 1995; Baker, 1993; Oldham et al., 1997). Unfortunately, most studies have addressed the effects of toxicological rather than sublethal concentrations of nitrates on amphibians. Amphibians exposed to nitrate concentrations ranging from 2.5 – 100 mg/L exhibit sublethal responses that are subtle compared to responses to toxic concentrations (Chapter 1, Table 1-2). Tadpoles of the Common toad (*Bufo bufo*) exposed to 50 - 100 mg/L nitrate exhibited delayed metamorphosis compared to unexposed tadpoles. Additionally, exposed toads exhibited unusual swimming movement, nostral deformities, and absent or deformed limbs (Xu et al., 1997). Cascades frog (*Rana cascadae*) tadpoles exposed to 3.5 mg/L nitrite also had delayed time to metamorphosis compared to controls (Marco and Blaustein, 1995). For tadpoles of some other anuran species, exposure to similarly sublethal concentrations is lethal. The variation in responses of tadpoles to nitrate exposure (at similar concentrations) indicates that there are species differences in susceptibility to perturbation by nitrate (Marco et al, 1999; De Solla et al., 2002).

Nitrate exposure has been shown to inhibit testosterone (T) synthesis in rodents (Panesar and Chan, 2000), and bulls (Zraly et al., 1997). Alligators exposed to nitrate at low concentrations

(1.65 mg/L) exhibited increased plasma steroid concentrations (*in vivo*) but testes exposed to directly to nitrate (*in vitro*) exhibited decreased testosterone synthesis (Guillette and Gunderson, unpubl. data). Thus, steroid synthesis in response to nitrate exposure can result in variable responses depending on the route of exposure. A mechanism for the disruption of steroidogenesis by nitrates has been proposed by Panesar and Chan (2000), and has been expanded by other researchers. Many body tissues contain nitric oxide synthase (NOS) enzymes that can convert nitrate into nitrite into NO (Forstermann et al., 1995; Szabo and Thiemermann, 1995; Wang and Marsden, 1995). In mammalian ovaries NO has also been shown to inhibit E_2 synthesis, decrease aromatase activity, and increase insulin-like growth factor 1 (IGF-1) synthesis (Erickson et al., 1989; Adashi, 1993; Van Voorhis et al., 1994; Olson et al., 1996; Samaras et al., 1996). Thus, nitrates, via conversion into NO within body tissues, could influence steroid and IGF-1 synthesis and action.

Insulin-like growth factor 1 (IGF-1), a polypeptide hormone, plays an integral role in normal reproduction, cell growth, tissue differentiation, and embryo development in amphibians as in reptiles, birds, and mammals (Pancak-Roessler and Lee, 1989; Smith et al., 2000; Werner and LeRoith, 2000; Allen et al., 2001). Few researchers have examined whether environmental contaminants, like nitrate, alters the synthesis or action of IGF-1, despite the importance of this hormone in normal vertebrate development and growth. Abnormally high expression of IGF-1 mRNA is associated with abnormal limb bud development and emergence in mammals and birds, and with cancer formation in somatic and reproductive tissues of humans and other vertebrates (Backlin et al., 1998; Allen et al., 2000; Grimberg and Cohen, 2000; Klotz et al., 2000). Increased IGF-1 expression, induced by estrogen exposure, induces fetal malformation, abnormal proliferation of uterine tissues, and formation of reproductive lesions in aquatic mammals (Backlin and Bergman, 1995; Backlin et al., 1998). Evidence presented in Chapter 4 demonstrated that E_2-treated ovariectomized *R. catesbeiana* exhibited increased plasma IGF-1 concentrations. Thus, E_2 and IGF-1 apparently have interrelated functions. Because nitrate has

been shown to alter E_2, it is probable that nitrate can also alter IGF-1. Despite findings of skeletal and tissue deformities in tadpoles after exposure to fertilizers (Jones et al., 1997; Lutz and Kloas, 1999), no study has examined whether nitrate alters reproductive steroids and IGF-1 in amphibians. Evidence presented within earlier chapters (Chapter 2 and 5) indicates that exposure to sublethal concentrations of nitrate alter reproductive tissues, increases plasma IGF-1 concentrations while decreasing ovarian steroid synthesis. Findings of nitrate-associated alteration of IGF-1 hormone and reproductive steroids might introduce a novel mechanism by which contaminants alter reproduction and growth, and contribute further insight into factors implicated in the apparent global decline of amphibians.

In this study, I examined the effects of sublethal concentrations of nitrate, administered *in vivo* to adult bullfrogs (*Rana catesbeiana*), on reproductive tissue, plasma steroid (E_2 and T) concentrations, and plasma IGF-1 concentration. Based on the aforementioned studies, I hypothesized that exposure of adult bullfrogs (*Rana catesbeiana*) to environmentally relevant concentrations of nitrate alters reproductive tissues, and plasma concentrations of sex steroids (E_2 and T) and IGF-1.

Materials and Methods

Animals

All work was conducted under IACUC approved guidelines (project #Z023). For this study, *R. catesbeiana* were chosen in lieu of *X. laevis* and *R. grylio*, which were examined in earlier chapters. Previous attempts to maintain wild-caught *R. grylio* in captivity demonstrated that they were highly stressed in captivity and were inappropriate subjects for the 10-day study outlined here. In contrast to *R. grylio*, *R. catesbeiana* were very adaptable and exhibited no observable stress in captivity. Additionally, *R. catesbeiana* are large-bodied frogs, which made it easier to collect a greater blood sample volume than in *X. laevis* and *R. grylio*. Oviduct growth response to normal physiological concentrations of E_2 and IGF-1 had already been established for *R. catesbeiana* (Chapter 4). Finally, normal concentrations of plasma steroids and IGF-1 had

already been determined for *R. catesbeiana* in (Chapter 4) and by Licht et al. (1984), and these studies served as references.

Adult *Rana catesbeiana* (30 females and 18 males) were purchased (Charles Sullivan Co. Inc., TN), maintained in 38 L tanks with 19 L of static flow, dechlorinated water at 26°C under a 12-h diurnal light/dark cycle. Tank water was changed and frogs were fed crickets every other day for the duration of the experiment. Frogs were randomly assigned to nitrate treatment groups. Each treatment group of either six female or six male frogs was separated into two replicate tanks each containing three frogs to avoid pseudo-replication. Frogs were maintained under these conditions for a 3-week acclimation period before the onset of the experiment.

Nitrate Treatments

Sodium nitrate ($NaNO_3$) was dissolved in deionized water to produce nitrate as nitrogen (NO_3-N) treatment concentrations of 0, 0.17, 1.65, 6.60, and 16.50 mg/L for females and 0, 1.65, and 6.60 mg/L for males. These concentrations are environmentally relevant and also include concentrations allowed in human drinking water according to standards established by the US EPA.

Rather than dissolving nitrate into tank water for whole-body exposure of *R. catesbeiana*, treatments were delivered as intra-abdominal injections. Unlike the fully aquatic *Xenopus laevis* (chapter 1), ranid frogs are semi-aquatic. Laboratory observations of *R. catesbeiana* have shown that they spend prolonged periods of time out of the water and display considerable stress (escape behavior and nostral abrasions) when denied access to basking platforms (T. Barbeau, pers. obs.). Whole-body exposure of frogs to aqueous nitrate would require removal of basking platforms to maintain continuous exposure to the treatments. Preventing *R. catesbeiana* from engaging in normal basking behavior could make it difficult to distinguish a stress response from other physiological responses to experimental treatments. If basking platforms were not removed from the treatment tanks, basking frogs would remain unexposed to aqueous nitrate treatments for variable time periods, making it impossible to determine their treatment times. To avoid these

confounding variables, nitrate treatments were dissolved in sterile, deionized water and delivered via intra-abdominal injections (0.5 ml) into the ventral paramedial surface as described by Wright and Whitaker (2001).

Before initiating the main experiment, 3 extra bullfrogs were given intra-abdominal nitrate injections (16.50 mg/L) daily for 1 week to confirm that the highest concentration and the delivery route were not toxic or lethal. Frogs exhibited normal behavior and feeding during the treatment period, and also for 2 weeks after treatments. Based on these preliminary findings, the nitrate concentrations and delivery route chosen were deemed safe for *R. catesbeiana* in this experiment.

Frogs were injected with nitrate every other day, for a total of 5 injections. This treatment regime was considered an acute exposure due to the direct route of administration and the relative brevity of treatment length. Intravenous injections of nitrate (at similar concentration), given to adult alligators have demonstrated that such brief exposure times are sufficient to elicit an endocrine response (Gunderson and Guillette, unpubl. obs). After treatment, frogs were anesthetized with MS-222 (1.5% 3-aminobenzoic acid ethyl ether, Aquatic Ecosystems, Orlando, FL), blood samples were collected via cardiac puncture, and the frogs were euthanized by dissection through the spinal cord followed by pithing. Plasma was frozen (-70°C) for E_2, T, and IGF-1 radioimmunoassay (RIA) analyses. The ovaries, oviducts, liver, spleen, and fat bodies were removed from females, and the testes, liver, spleen, and fat bodies were removed from males, and tissues were weighed to determine wet mass among treatment groups. Cross sections of oviducts (ampulla region) were fixed in 4% paraformaldehyde (4°C; 48 h) followed by rinse and storage in 75% ethanol for subsequent histological analyses. The oviducts were dehydrated in a graded series of ethanol changes, embedded in paraffin, serially sectioned on a rotary microtome (7 μm), stained with hematoxylin and eosin, and examined using light microscopy. To ascertain oviductal proliferation among treatment groups, an ocular micrometer was used to make

10 morphological measurements on five tissue sections per frog, for each of the following oviductal parameters: epithelial cell height, endometrial thickness, endometrial gland height, and endometrial gland width. Endometrial gland height and width measurements were used to calculated total gland surface area.

Steroid Radioimmunoassay (RIA) Procedures and Validations

Validation of steroid RIA was accomplished with pooled plasma dilutions for females and males (25, 50, 100, 200, and 300 μL for E_2 and 6.25, 25, 50, 100, and 200 μL for T), and borate buffer was used to bring all samples up to 200 μL total volume. For internal standards, 100 μL aliquots of steroid-stripped plasma were spiked with standards for E_2 and T (1.06, 3.125, 6.25, 12.5, 25, 50, 100, 200, 400, and 800 pg/tube). Plasma dilution curves and internal standard curves were compared with E_2 and T standard curves. Before analyses, hormone concentrations were log10-transformed and ANCOVA was used to verify homogeneity of slopes with the standard curves for plasma dilutions and for internal standards.

Internal standards and plasma dilutions were parallel to the standard curve for E_2 in females (ANCOVA; $F = 0.03$, $P = 0.73$ and $F = 0.01$, $P = 0.91$, Fig. 6-1A) and males (ANCOVA; $F = 0.001$, $P = 0.99$ and $F = 1.86$, $P = 0.22$, Fig. 6-2A), and for T in females (ANCOVA; $F = 0.001$, $P = 0.99$ and $F = 0.08$, $P = 0.79$; Fig. 6-1B) and males (ANCOVA; $F = 0.003$, $P = 0.96$ and $F = 0.37$, $P = 0.56$, Fig. 6-2B). For females and males, average recoveries after plasma extractions for E_2 were 93.9% and 85.0%, for T were 87.9% and 94.1%, respectively. Final media hormone concentrations were changed to reflect these losses. Finally, E_2 and T validation RIA intraassay variance averaged 2.87% and 4.88%; and RIA samples for E_2 and T were each run in 2 assays having an average intraassay variance of 1.53% and 1.23%, and interassay variance of 7.84% and 2.56%, respectively.

RIAs were performed for E_2 and T on plasma samples using validated procedures. Based on RIA validations, 50, 6.25, and 50 μL of female plasma were used while 300, 12.5, and 50 μL

of male plasma were used for E_2, T, and IGF-1 analyses, respectively. Duplicate plasma samples were extracted twice with ethyl-ether, air-dried, and reconstituted in 100 uL borate buffer (0.05 M; pH 8.0). Antibody (Endocrine Sciences) was added at a final concentration of 1:25,000 for T and 1:55,000 for E_2. Radiolabeled steroid ([2,4,6,7,16,17-^{3}H] estradiol at 1 mCi/ml; [1,2,6,7-^{3}H] testosterone at 1 mCi/mL; Amersham Int., Arlington Heights, IL) was added at 12,000 cpm per 100 µL for a final assay volume of 500 µL. Interassay variance tubes were prepared from two separate pools of media and plasma for T and E_2. Standards for T and E_2 were prepared in duplicate at 0, 1.06, 3.13, 6.25, 12.5, 25, 50, 100, 200, 400, and 800 pg/tube. Assay tubes were vortexed and incubated at 4°C for 24 h. Bound-free separation was performed using a mixture of 5% charcoal and 0.5% dextran, pulse-vortexing, and centrifuging tubes (1500g, 4°C, 30 min). Supernatant was added to 5 mL of scintillation cocktail, and counted on a Beckman scintillation counter. For E_2 and T RIA, male and female plasma samples were analyzed within a single assay with an average intraassay variance of 4.02% and 5.42%, respectively.

Insulin-Like Growth Factor-1 (IGF-1) RIA Procedures and Validations

Validation of IGF-1 RIA was accomplished with 300 µL of pooled plasma dilutions for females and males. Plasma was extracted in polypropylene tubes with acid-ethanol (12.5% 2 N HCl, 87.5% ethanol; 800 µL) to dissociate IGF binding proteins from the IGF-1 molecules and to precipitate globular proteins (Daughaday et al., 1980; Crain et al., 1995). After 30 min incubation (room temperature) and 10-min centrifugation (2500×G; 4°C), the supernatant was aliquoted to produce plasma equivalents of 12.5, 25, 50, 100, 200, and 300 µL. Volume of the plasma dilutions was brought to 200 µL with acid-ethanol prior to air-drying. Plasma dilutions were compared with 0, 39, 156, 313, 625, 1000, 1250, 2500 pg of human recombinant IGF-1 standard (National Hormone and Pituitary Program, Torrance, CA 90509). Validation samples were examined by IGF RIA procedures as described for experimental analyses below.

Plasma extraction efficiencies were determined by adding 100 μL iodinated IGF-1 (15,000 cpm) to 100 μL of pooled plasma samples, extracting with acid-ethanol, air-drying, and reading samples on a Beckman 5500B gamma counter to determine the iodinated hormone remaining. The extraction efficiency of plasma was 77.0%, and all sample concentrations were corrected for this loss. Validation of plasma dilutions for IGF-1 was accomplished in a single assay having an intraassay variance of 2.27%.

Plasma dilutions were parallel to the standard curve for IGF-1 in females (ANCOVA; $F = 0.01$, $P = 0.91$, Fig. 6-1C) and males (ANCOVA; $F = 5.14$, $P = 0.10$, Fig. 6-2C). For IGF-1 validation RIA and sample RIA were each run in a single assay with an average intraassay variance of 2.32% and 4.18%, respectively. For IGF-1, average recoveries after plasma extractions for females and males were 78.0% and 76.0%, respectively.

IGF-1 RIA was performed as described by Crain et al. (1995) and Guillette et al. (1994). The National Hormone and Pituitary Program (Torrance, CA 90509) supplied human recombinant IGF-1 standard (9.76 to 2500 pg/tube), and human IGF-1 antisera (Lot # AFP4892898, 1:400,000 final dilution). The antiserum had less than 1.0% cross-reactivity with human IGF-II. Iodinated IGF-1 label ($IGF\text{-}1^{I125}$ sp act 2000 Ci/mmol; 16,000 cpm/tube) and Amerlex-M donkey anti-rabbit secondary antibody (code RPN510, 500 μl/tube) were supplied through from Amersham International (Arlington Heights, IL). Buffer reagents were purchased from Fisher Chemical Co. (Pittsburgh, PA). Briefly, samples were aliquoted into polypropylene tubes, extracted with 400uL acid-ethanol, and incubated 30 min prior to centrifugation (2500×G; 4°C; 10 min). For each sample, supernatant (100 μL) was pipetted into duplicate polypropylene tubes and air dried. IGF-1 standards were prepared in duplicate with 100 μL of known concentrations of human recombinant IGF-1 standard (ranging from 9 - 2500 pg/tube), and 300 μL RIA buffer (200 mg/L protamine sulfate, 4.14 g/L sodium phosphate monobasic, 0.05% TWEEN 20, 0.02% sodium azide, 3.72 g/L EDTA) added to each tube. Air-dried samples were

reconstituted with 350 μL RIA buffer and vortexed. To each sample, 50 μL IGF-1 antibody (human IGF-1 antisera, UB3-189) was added at a 1:10,000 final dilution. After adding 100 μL of iodinated IGF-1 label (I^{125}-IGF-1), with ~15,000 CPM, samples were vortexed and incubated overnight at 4°C. Separation of bound and free IGF-1 was accomplished by incubating samples for 10 min with 500 μL of secondary antibody (Amerlex-M donkey anti-rabbit secondary antibody, code RPN.510 obtained from Amersham International) at a final dilution of 1:10,000. Sample tubes were centrifuged (2500×G; 4°C; 10 min) to separate the secondary antibody, which is bound to the primary antibody and ligand. The supernatant was decanted and the pellet counted on a Beckman 5500B gamma counter. Female and male plasma samples were run in one assay having an average intraassay variance of 2.32%.

Statistics

Concentrations of E_2 and T were estimated from raw data using Microplate Manager software (Microplate Manager III, BioRad Laboratories, Inc., Hercules, CA, 1988). Unpaired, two-tailed t-tests revealed no significant differences ($P > 0.05$) in mean hormone concentration or tissue mass between females and males from replicate tanks per treatment group. Additionally, no significant differences were detected in female oviduct morphology parameters between replicate groups ($P > 0.05$). Thus, for final analyses, parameter measurements from females and males in replicate treatment tanks were lumped together for a total sample size of six frogs per treatment group.

Hormone concentrations among treatment groups were analyzed by ANOVA followed by Fishers Protected LSD contrasts. For E_2 concentrations in females and males, and for T concentrations in females, variances were unequal among treatment groups; therefore, ANOVA was followed by Tamhane contrasts. Oviduct, liver, spleen, and fat body masses among treatment groups were analyzed by ANCOVA using body mass as a covariate. Lastly, morphological tissue measurements of oviducts were analyzed by ANOVA followed by Tamhane contrasts to account

for unequal variance among treatment groups. Statistical analyses were performed using SPSS software (v. 10, SPSS Inc., Chicago, IL, 1999) with $\alpha = 0.05$.

Results

Oviduct Weights

For females, there was no significant difference in wet tissue mass, with body weight as a covariate, among treatment groups for liver (ANCOVA; $F = 1.25$, $P = 0.32$), ovaries (ANCOVA; $F = 0.53$, $P = 0.72$), oviducts (ANCOVA; $F = 0.76$, $P = 0.56$), or fat bodies (ANCOVA; $F = 0.54$, $P = 0.70$). For males, there were no significant differences in wet tissue mass among groups for liver (ANCOVA; $F = 1.73$, $P = 0.22$), testes (ANCOVA; $F = 1.34$, $P = 0.29$), or fat bodies (ANCOVA; $F = 3.68$, $P = 0.06$).

Plasma Steroid and IGF-1 Concentrations

Plasma E_2 and IGF-1 concentrations were increased in females exposed to 1.65, 6.60, and 16.50 mg/L nitrate compared to females in control and 0.17 mg/L groups (ANOVA; $P < 0.001$, Fig. 6-3A,C). Plasma T concentrations in all females exposed to nitrate (0.17, 1.65, 6.60, and 16.50 mg/L) were higher than in control females (ANOVA; $P < 0.001$, Fig. 6-3B). Rather than a typical dose-response curve, plasma concentrations of E_2 and T displayed a non-monotonic dose response curve. For both steroids, and initial increase in plasma steroids was seen at 1.65 followed by a decrease at 6.60 mg/L and an increase at 16.50 mg/L. Plasma E_2 was elevated in male frogs exposed to 1.65 mg/L but not to 0 mg/L and 6.60 mg/L nitrate. Plasma T was similar in males among treatment groups. In males, plasma IGF-1 was higher with exposure to both 1.65 mg/L and 6.60 mg/L nitrate compared to control males (ANOVA; $P < 0.001$, Fig. 6-4).

Oviduct Morphometrics

The epithelial layer bordering the oviductal lumen is a continuous and convoluted layer of ciliated, cuboidal cells with darkly staining basal nuclei and an apical brush-border of cilia

projecting into the lumenal space. The epithelial cilia are relatively short (< 15 μm), uniform, and numerous.

The epithelial cell heights were lowest in females exposed to 6.60 mg/L, and greatest in those exposed to 0.17 mg/L and 1.65 mg/L (Fig. 6-5A). However, epithelial cell heights were similar in females exposed to 0 and 16.50 mg/L nitrate. Endometrial thickness was significantly lower in females exposed to all nitrate doses with the lowest measures in the 16.50 mg/L group (Fig. 6-5B). Overall, endometrial gland area (μm^2) was lower in females exposed to nitrate (Fig. 6-5C).

Discussion

In recent decades, high concentrations of nitrate in watersheds of North American might reflect an increase in nitrogen input from many sources including runoff of nitrogenous fertilizers. Increased nitrogen contamination of aquatic ecosystems poses severe health consequences to domestic and wild animals, and perhaps even humans. Rodents exhibit altered steroidogenesis, both *in vivo* and *in vitro*, when exposed to nitrate (Panesar and Chan, 2000). Rodents consuming 8.25 mg/L nitrate-as-as-nitrogen in their drinking water, for 4 weeks, exhibited decreased plasma T concentrations (*in vivo*). Testes of rodents incubated for 1 h with 1726 mg/L nitrate (*in vitro*) exhibited decreased T synthesis (Panesar and Chan, 2000). Nitrate exposure also inhibits steroid synthesis in other mammals, including humans (Wang and Marsden, 1995) and bulls (Zraly et al., 1997). Bulls consuming potassium nitrate (100 - 250 g) in their feed for 30 days exhibited a decreased testicular T synthesis (*in vitro*), in response to gonadotropin stimulation (Zraly et al., 1997). The proposed mechanism for nitrate-associated endocrine disruption steroid involves the following complex pathway. Nitrate is converted into nitrite by bacteria within the mouth and gastrointestinal tract (Painter, 1975; Mayer, 1997). Nitrite is then converted into NO by various isoforms of nitric oxide synthase (NOS) found in body tissues (Kukovetz et al., 1987; Kleinert et al., 1995; Ellis et al., 1998). The NO is a highly diffusible compound that can enter most cells and

tissues to induce a variety of physiological effects. NO reacts readily with metals like iron and iron-sulfur centers within heme-containing enzymes, such as all the enzymes of the P450 superfamily. Most interactions of NO result in inhibition of these enzymes, with the exception of guanylate cyclase, that is stimulated by NO (McDonald and Murad, 1995). The mitochondrial enzymes P450 side-chain cleavage ($P450_{scc}$) and 3β-hydroxysteroid dehydrogenase (3β-HSD) contain heme subgroups that are inhibited by NO (Delaforge et al., 1995; Del Punta et al., 1996). These enzymes are necessary to convert free cholesterol within the mitochondria into progesterone (P4), the steroid precursor to T. After T is synthesized it is converted into E_2 by aromatase enzymes (Chapter 1, Fig. 1-1). Additionally, NO interferes with steroidogenic acute regulatory (StAR) protein necessary for transport of free cholesterol into the mitochondria (Wang and Marsden, 1995; Stocco, 1999). Thus, NO likely disrupts steroidogenesis by inactivation of P450 and 3β-HSD enzymes and interference with StAR protein (VanVoorhis et al., 1994, 1995; Panesar, 1999; Stocco, 1999; Panesar and Chan, 2000).

Nitric oxide inhibits steroid synthesis and aromatase gene expression within the gonads of humans, rodents, pigs, and cows (Adashi, 1993; VanVoorhis et al., 1994; Olsen et al., 1996; Snyder et al., 1996; Srivastava et al., 1997; Basini et al., 1998). Despite findings of nitrate-associated endocrine disruption in other animals, no previous study examines whether nitrate alters endocrine function in amphibians. Most studies on the effects of sublethal and lethal nitrate concentrations on amphibians have focused on tadpoles. Nitrate exposure reportedly alters growth, development, feeding, swimming, metamorphosis, and mortality rates of tadpoles both in the field and the laboratory. Only one study reports the effects of lethal nitrate concentrations on adult amphibians (Oldham et al., 1997). No published investigations have reported the effects of exposure to sublethal nitrate concentrations on endocrine function in amphibians.

This study presents the first findings of an association between altered plasma steroids and IGF-1 and oviductal atrophy in *R. catesbeiana* after acute exposure to sublethal nitrate

concentrations. Furthermore, nitrate exposure is associated with endocrine disruption in *R. catesbeiana* at concentrations that are environmentally relevant and lower than the MCL allowed in drinking water. In female *R. catesbeiana*, plasma E_2 and T concentrations did not exhibit a typical dose-response increase with increasing nitrate concentrations. Rather, the steroid response more closely resembled an inverted U-shaped non-monotonic dose response (NMDR) for nitrate concentrations below 16.50 mg/L. E_2 and T concentrations are increased at 1.65 mg/L nitrate but then decreased with a slightly higher nitrate concentration of 6.60 mg/L. At 16.50 mg/L, E_2 and T concentrations increase again. A similar NMDR was seen for male *R. catesbeiana* at similar nitrate concentrations. The NMDR curve is commonly seen with studies of endocrine disruption and reflects greater sensitivity of steroid receptors to endocrine-disrupting contaminants (EDCs) present at extremely low concentrations. At extremely low EDC concentrations, many steroid receptors remain unoccupied and inactive. Only slight increases in the EDC concentrations can dramatically increase receptor binding up to 100 percent. Afterwards, any further increases in EDC concentrations cannot lead to a greater steroid response because all the steroid receptors are bound (vom Saal et al., 1997; Welshons et al., 2003).

In mammals nitrate exposure (*in vivo*) is associated with decreased plasma and gonadal steroid concentrations (Panesar and Chan, 2000; Zraly et al., 1997). In *X. laevis*, nitrate exposure (*in vivo*) is associated with a significant decrease in gonadal steroid synthesis (Chapter 2). These findings in *X. laevis* were similar to steroid responses of mammals exposed to nitrate through consumption of nitrate in water (Panesar and Chan, 2000) and feed (Zraly et al., 1997). *Rana grylio* ovarian follicles exposed to nitrate and nitrite also exhibited decreased steroid synthesis (*in vitro*). The decreased steroid concentrations (*ex vivo* and *in vitro*) reported for *X. laevis* and *R. grylio* (Chapters 2 and 5) conflict with the findings of increased plasma steroid concentrations in *R. catesbeiana* with nitrate exposure (*in vivo*). In this study, the use of intra-abdominal nitrate injections might have influenced the steroid hormone response by a mechanism other than that seen with exposure of the gonad (*in vitro*) or ingestion of nitrate (*in vivo*). For example, studies in

alligators have shown that nitrate exposure (via an intravenous injection) stimulated increased plasma steroid concentrations; however, direct incubation of the testes with nitrate (*in vitro*) causes decreased gonadal steroid synthesis (Guillette and Gunderson, unpubl. obs.). Thus, the route of nitrate exposure apparently influences steroid responses.

The effects of *in vivo* nitrate exposure likely involve a integrated physiological response that is highly complex compared to the responses of isolated tissues cultured with nitrate (*in vitro*). Circulating hormone concentrations can change in response to signals from the hypothalamic-pituitary-gonadal (HPG) axis, in response to interaction with plasma binding proteins, and also in response to steroid metabolism and clearance by the liver (Chapter 1, Fig. 1-1). Gonadal steroid synthesis (*in vitro* or *ex vivo*) was demonstrated in earlier studies to decrease with nitrate exposure; however, nitrate exposure can influence plasma steroid concentrations (*in vivo*) by affecting other organs. A severe depression of liver cytochrome P450 enzyme activity could decrease steroid metabolism and clearance, and contribute to elevated plasma steroid concentrations (Fig. 2-7). One recent study has shown that rats administered organic nitrate exhibit decreased activity of hepatic P450 enzymes within 24 h of exposure (Minamiyama et al., 2004). Zraly et al. (1997) reported that bulls fed potassium nitrate exhibited increased concentration of plasma bile acids, decreased progesterone metabolism, and abnormal liver morphology that collectively signify impaired liver function in response to nitrate exposure. These findings also support a pathway, proposed here, for the increase in plasma steroid and IGF-1 concentrations observed in *R. catesbeiana* in response to nitrate exposure (*in vivo*). The liver degrades the majority of nitrate consumed and circulating in the body. Nitrate exposure has been shown to decrease activity of hepatic cytochrome P450 enzymes, perhaps via formation of NO. Since hepatic P450 enzymes are necessary to metabolize and clear steroid from circulation, inhibition of these enzymes with nitrate exposure can contribute to elevated concentrations of circulating steroid. A hepatic response to nitrate exposure is further supported by findings reported by de Caceres et al. (2003). Nitrate and nitrite exposure can influence NO formation in

the hypothalamus. NO stimulates hypothalamic synthesis of growth hormone-releasing hormone (GHRH) causing pituitary secretion of growth hormone (GH) into the bloodstream (de Caceres et al., 2003). Circulating GH stimulates the liver to synthesize and secreted IGF-1 into the bloodstream. In this manner, nitrate exposure could activate the hypothalamic-pituitary-hepatic (HPH) axis to influence plasma IGF-1 concentrations (Chapter 2, Fig. 2-7).

In future studies the liver should be examined, in addition to the gonadal steroidogenesis and plasma hormone concentrations, with different routes of nitrate exposure because studies reported here indicate that the route of nitrate exposure, as well as the endpoint measured, will influence the perceived endocrine response.

The increase in plasma IGF-1 concentrations in *R. catesbeiana* with nitrate exposure might occur in response to apoptosis within oviductal cells. The stimulatory effect of IGF-1 on cell growth can counteract apoptosis in reproductive tissues (Ponderato et al., 2000). Increased plasma IGF-1 concentrations might also occur in response to an initial inhibitory effect of nitrate on ovarian steroid synthesis. In mammalian ovaries, IGF-1 can also counteract the inhibitory effects of NO on steroid synthesis by stimulating increased P_4, E_2, aromatase, and steroidogenic acute regulatory (StAR) protein synthesis (Devoto et al., 1999; LaVoie et al., 1999; Sekar et al., 2000; Iniguez et al., 2001; Les Dees et al., 2001). Thus it is possible that plasma IGF-1 concentrations increased with nitrate exposure to counteract decreased gonadal steroid synthesis. Over the 10-day study, elevated plasma IGF-1 concentrations in response to nitrate might have been sufficient to restore and even elevate plasma steroid concentrations during this period. Thus, it is possible that the increase in plasma IGF-1 concentration with nitrate exposure was a compensatory response to increased gonadal NO synthesis (Chapter 2, Fig. 2-7). However, NO and steroid synthesis within the gonads of *R. catesbeiana* was not measured in this study, and this pathway remains purely speculative.

Nitrate exposure of female *R. catesbeiana* is associated with atrophy of the oviductal endometrial layer and gland surface area. Nitrate has been shown to induce apoptosis or cell death

in a variety of tissue types including the tail of metamorphosing tadpoles, and the brain, testis, and gastric mucosa of mammals (Kashiwagi et al., 1999; Peltola et al., 2001; Pant and Srivastava, 2002; Tari et al., 2003). The proposed mechanism for nitrate-induced apoptosis involves, again, NO activity. NO can target nearly all cell components including proteins, carbohydrates, lipids, and nucleic acids. NO can induce cell death by inhibiting heme-containing enzymes that are vital in cell growth including energy metabolism and the synthesis of purines and pyrimidines needed for nucleic acid production (Lincoln et al., 1995). NO can also induce a cytotoxic response in tissues by reacting with superoxides to form the free radical peroxynitrite. Peroxynitrite a highly destructive compound that damages cells and exacerbates inflammatory responses (Bastian et al., 2002). In *R. catesbeiana*, the observed atrophy of the endometrium and glands in response to nitrate exposure might have occurred through NO-inhibition of heme-containing enzymes involved in DNA synthesis or through a cytotoxic production of peroxynitrite. It must be mentioned that nitrite, by itself, is highly toxic and might have induced oviductal atrophy in response to the direct injection of nitrate into the abdominal cavity. However, the effect of nitrite exposure on amphibian oviductal growth is unknown.

Further studies are necessary to elucidate the pathway by which nitrate interferes with oviduct morphology and with steroid and IGF-1 hormones in amphibians. It is unknown whether nitrate-associated oviductal atrophy and endocrine disruption in *R. catesbeiana* occurs through a NO-dependent pathway. First, it is necessary to determine whether NOS and NO are found within amphibian reproductive tissues, and whether they change expression with nitrate exposure. The use of NO donors and inhibitors might uncover how nitrate alters gonadal steroid synthesis in amphibians. If steroid synthesis is inhibited in gonadal tissue exposed simultaneously to nitrate and NO agonists, but steroid synthesis is restored with the addition of NO inhibitors, then nitrate-associated endocrine disruption in amphibians likely operates through a NO-dependent pathway. However, if gonadal steroid synthesis with nitrate exposure is unaffected by the addition of NO donors and inhibitors, a different pathway of nitrate-associated endocrine disruption likely occurs

in amphibians. Similarly, if NO expression is increased in atrophied oviductal tissue exposed to nitrate but is decreased in unexposed tissue, then a NO-pathway for apoptosis exists in amphibian oviducts.

There is another possible pathway for nitrate-associated endocrine disruption that has scarcely received attention and should be investigated further. Nitrate exposure might not alter steroids and IGF-1 solely through direct gonadal effects, but indirectly through effects on the hypothalamus or the pituitary. The pituitary, brain, and spinal cord contain neuronal NOS (nNOS) and the central nervous system also contains brain constitutive NOS (bNOS) (McDonald and Murad, 1995). Nitrate exposure might upregulate NOS enzymes thereby stimulating NO synthesis within the brain. Subsequently, NO might alter hypothalamic gonadotropin-releasing hormone (GnRH) and growth hormone releasing hormone (GHRH) secretion and pituitary gonadotropin (LH and FSH) and growth hormone (GH). The tropic hormones FSH and LH influence gonadal steroid synthesis, and GH influences hepatic and ovarian IGF-1 synthesis (Chapter 2, Fig. 2-7). This pathway remains an intriguing topic for further investigation.

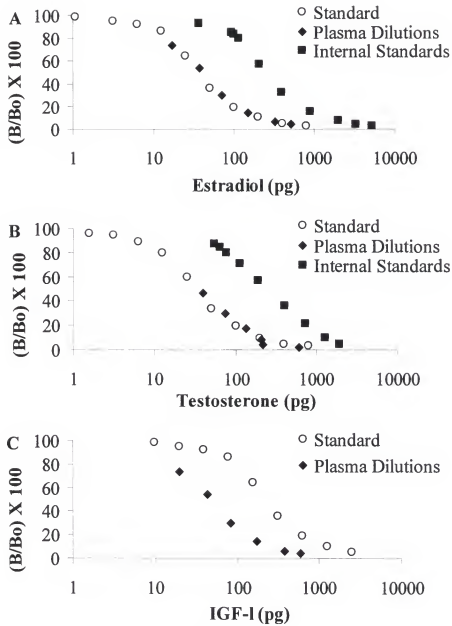

Figure 6-1. Biochemical validation of female *Rana catesbeiana* plasma for RIA. A. For 17β-estradiol internal standards and plasma dilution curves were parallel to the estradiol standard curve (ANCOVA; F = 0.03, *P* = 0.73; and F = 0.01, *P* = 0.91). B. For testosterone internal standards and plasma dilution curves were parallel to the testosterone standard curve (ANCOVA; F = 0.001, *P* = 0.79; and F = 0.08, *P* = 0.79). C. For insulin-like growth factor-1 (IGF-1) the plasma dilution curve was parallel to the IGF-1 standard curve (ANCOVA; F = 01, *P* = 0.91).

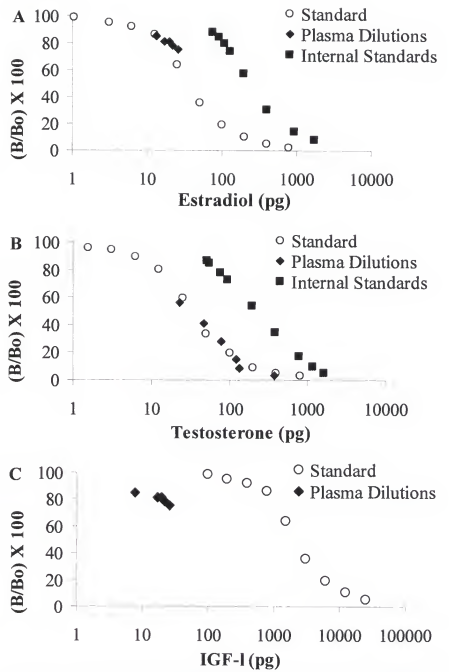

Figure 6-2. Biochemical validation of male *Rana catesbeiana* plasma for RIA. A. For 17β-estradiol internal standards and plasma dilution curves were parallel to the estradiol standard curve (ANCOVA; F = 0.001, $P = 0.99$; and F = 1.86, $P = 0.22$). B. For testosterone internal standards and plasma dilution curves were parallel to the testosterone standard curve (ANCOVA; F = 0.003, $P = 0.96$; and F = 0.37, $P = 0.56$). C. For insulin-like growth factor-1 (IGF-1) the plasma dilution curve was parallel to the IGF-1 standard curve (ANCOVA; F = 5.14, $P = 0.10$).

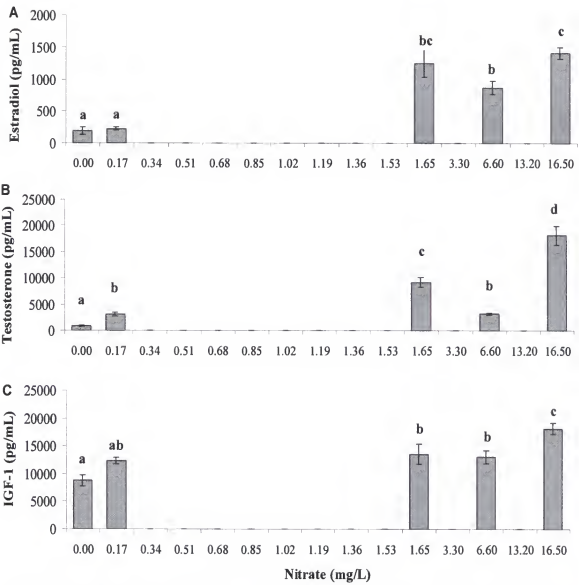

Figure 6-3. Mean plasma hormone concentrations of female *Rana catesbeiana* after nitrate (NO_3-N) treatments. Data presented as means ± SEM. Different letters above bars indicate significant differences for A. 17β-estradiol (ANOVA; N = 6, $P < 0.001$), B. testosterone (ANOVA; N = 6, $P < 0.001$), and C. insulin-like growth factor-1 (IGF-1) (ANOVA; N = 6, $P < 0.001$).

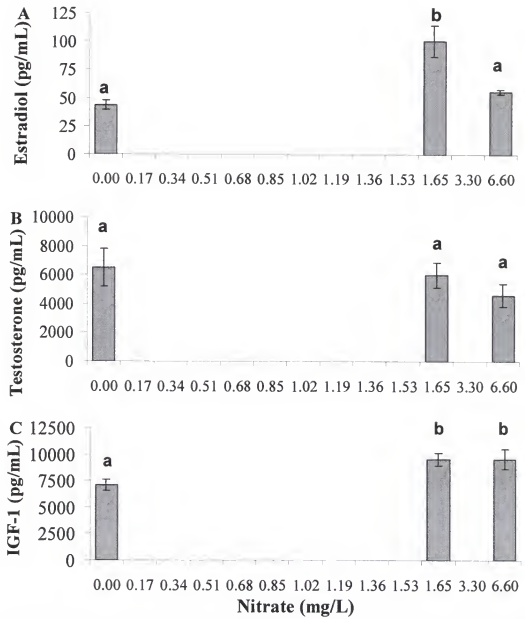

Figure 6-4. Mean plasma hormone concentrations of male *Rana catesbeiana* after nitrate (NO_3-N) treatment. Data presented as means ± SEM. Different letters above bars indicate significant differences for A. 17β-estradiol (E_2) (ANOVA; N = 6, $P < 0.001$) and C. insulin-like growth factor-1 (IGF-1) (ANOVA; N = 6, $P = 0.03$). B. No significant differences were detected in plasma testosterone (T) among treatment groups (ANOVA; $P = 0.41$)

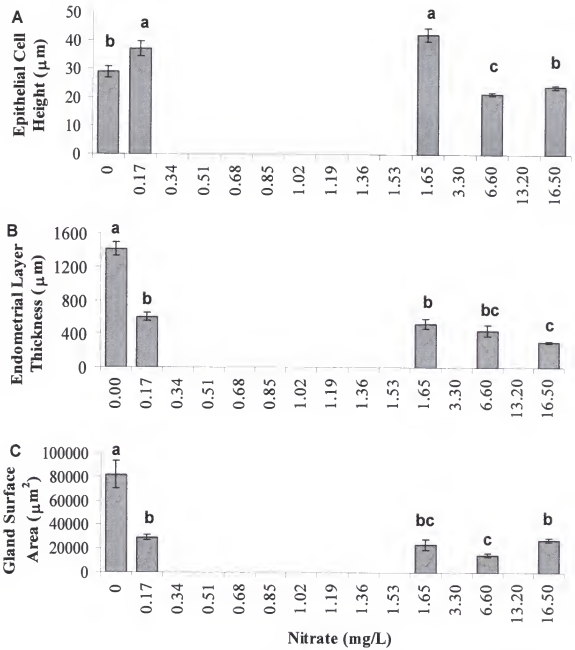

Figure 6-5. Oviduct morphology measurements for female *Rana catesbeiana* after nitrate treatment. Data presented as means ± SEM. Different letters above bars represent significant differences among means for A. epithelial cell height (ANOVA; $P < 0.001$), B. endometrial layer thickness (ANOVA; $P < 0.001$), and C. gland surface area (ANOVA; $P < 0.001$).

CHAPTER 7
CONCLUSIONS

Seasonal Plasma Steroids and IGF-1, and Reproductive Tissue Growth

Plasma steroid and IGF-1 concentrations, in addition to reproductive tissue growth, exhibited a clear pattern of seasonal changes in adult, female *R. grylio*. Growth of reproductive tissues (ovaries and oviducts) was positively correlated with increasing plasma E_2 and T concentrations. The seasonal pattern of changes in reproductive condition in *R. grylio* varied only slightly with published reports for other temperate-breeding ranid frogs (Licht et al., 1983; Moore and Deviche, 1988; Pancharatna and Saidapur, 1992). Licht et al. (1983) reported for *R. catesbeiana* that gonadal-somatic index (GSI) and oviductal weights, in addition to plasma steroid and gonadotropin concentrations, increased between May and July, and declined in August. In *R. grylio* from Alachua, county, Florida, seasonal changes in GSI and ovarian maturation in *R. grylio* closely match descriptions of seasonal breeding condition and behavior of *R. grylio* in Southern Georgia (Lamb, 1984; Wright, 1932), other regions of North-Central Florida (Wood et al., 1998), and in the Everglades of South Florida (Ligas, 1960). In South Florida (Lygas, 1960) and in South Georgia (Lamb, 1984), GSI and ovarian maturation in *R. grylio* increased between April and July, and regressed between August and March. Collectively, these findings indicate little variation in seasonal reproductive condition for *R. grylio* among these geographic locations.

Very little is known about seasonal changes in circulating IGF-1 concentrations in amphibians. A seasonal pattern of plasma IGF-1 concentrations has been reported only for *Bufo woodhousei* (Pancak-Roessler and Lee, 1990). In *B. woodhousei*, plasma IGF-1 concentrations peaked during the reproductive period (May and June) and decreased during the non-reproductive period (August and December). In *R. grylio*, plasma IGF-1concentrations exhibited a similar

pattern of seasonal change. Additionally, plasma IGF-1 concentrations in *R. grylio* were highly correlated to air temperature and fat body weights. As air temperature increased and fat body weights decreased, plasma IGF-1 concentrations increased. Presumably, fat stores were depleted due to increased fat metabolism in the liver, which can provide valuable energy to fuel tissue growth and gamete maturation during the breeding period. In ectotherms, food intake and metabolic processes typically increase with increasing internal and environmental temperatures (Lillywhite et al., 1973; Larson, 1992; Rome et al., 1992). Hepatic fat metabolism was not measured in *R. grylio* during this study; therefore this remains a speculative but interesting topic for further research. Nutritional status is known to influence plasma IGF-1 in ectotherms (Crain et al., 1994; Thissen et al., 1994), suggesting the prediction that plasma IGF-1 concentrations would increase with declining fat body weights in *R. grylio*. Overall, these findings suggest that environmental cues and nutritional status regulate IGF-1 in frogs.

Months of increased plasma IGF-1 overlapped with months of increased ovarian and oviductal weights, and also with increased plasma E_2 and T concentrations. However, no significant correlation was detected between IGF-1 and steroid concentrations, or between IGF-1 and ovarian and oviductal weights. The association between plasma steroids and IGF-, and the role of these hormones in regulating growth of reproductive tissues is still unclear. Although synergy between E_2 and IGF-1 stimulates greater oviductal growth than either hormone does alone in mammals (Murphy and Murphy, 1994) it is unknown whether these hormones stimulate oviductal growth similarly in *R. grylio*. During the reproductive periods, synthesis of IGF-1 in *R. grylio* might be elevated for incorporation of this growth factor into mature eggs, and for stimulation of oviductal secretions that function in egg transport and subsequent fertilization (Low et al., 1976; Guillette et al., 1996; Olsen and Chandler, 1999). Unlike IGF-1, plasma steroid concentrations showed a strong positive correlation with reproductive tissue weights in *R. grylio*. The pattern of increased oviductal growth with increasing plasma steroid concentrations

described for *R. grylio* matches the increase in oviduct growth in *R. catesbeiana* with E_2 treatments reported in Chapter 4.

The Effects of IGF-1, E_2, and Nitrate on Oviduct Growth

In Chapter 4, ovariectomized *R. catesbeiana* were examined for the effects of growth factors, E_2, and combined E_2/IGF-1 on various parameters of oviductal growth: oviductal weight, epithelial cell height, endometrial thickness, and endometrial gland surface-area. In summary, E_2-treated *R. catesbeiana* exhibited greater oviductal growth compared to IGF-1- and EGF-treated frogs, indicating that IGF-1 and EGF alone did not stimulate oviductal growth of these parameters. Additionally, E_2/IGF-1-treated frogs did not exhibit greater oviductal growth, for most parameters, than did frogs given E_2 alone, indicating thatE_2/IGF-1 did not act synergistically to stimulate greater oviductal growth compared to either treatment alone. The only exception to this pattern was E_2/IGF-1-treated frogs had a greater oviductal epithelial cell height compared to all other treatments, indicating synergy between the hormones.

The absence of a growth response in the oviductal endometrial layer and glands to either EGF or IGF-1 alone, and the absence of a synergistic growth response to E_2/IGF-1 treatment distinguished *R. catesbeiana* from reptilian and mammalian species examined to date using similar techniques. Some reptiles and mammals exhibit oviductal growth in response to treatment with IGF-1 or EGF alone (Murphy and Ghahary. 1990; Cox and Guillette, 1994). Additionally, oviductal growth in these mammals is greater when treated with combined E_2/IGF-1 than with either growth factor alone, indicating hormonal synergy of E_2 and IGF-1 (Murphy and Murphy, 1994). Although plasma IGF-1 and EGF have been identified in several amphibian species (Daughaday et al., 1985; Pancak-Roessler and Lee, 1990), the oviduct of *R. catesbeiana* apparently does not proliferate significantly in response to stimulation by these hormones. Several factors may explain the absence of an oviductal growth response to either growth factor, or combined E_2/IGF-1, in *R. catesbeiana*. First, the IGF-1 doses selected for this study may have been insufficient to elicit an oviductal growth response in *R. catesbeiana*. Future studies should

investigate several doses of IGF-1, both higher and lower, to determine if any dose is capable of stimulating oviductal growth in ovariectomized *R. catesbeiana*. Further, the delivery system may need to be examined – we used implanted pellets, which have worked in other species, but studies examining injections could also provide important information. Second, it is possible that the oviduct must first be "primed" by E_2-exposure prior to IGF-1 exposure to become sensitive to the effects of IGF-1 (Figure 7-1). This priming of oviductal tissue could involve upregulation of estrogen receptors, such as ERα, and IGF-1 receptors (IGF-1R). This hypothesis is supported by studies showing that ERα must be present for IGF-1 to induce effects (Klotz et al., 2000). Additionally, Clark et al. (1997) demonstrated that E_2 stimulates a proliferative response in reproductive tissues *in vitro* by upregulating IGF-1R expression, which increases the tissue's response to circulating IGF-1. Based on these findings, it is possible that an increase in circulatory E_2 increases receptor-dependent tissue sensitivity to the growth-promoting effects of IGF-1 without necessarily requiring an increase in circulatory IGF-1 (Figure 7-1). This seems to be supported by the increased oviductal epithelial cell height with E_2/IGF-1-treated *R. catesbeiana*.

Another possible explanation is that oviductal sensitivity to these growth factors represents a relatively recent evolutionary change in reptilian and mammalian reproductive physiology. However, more amphibian species should be examined, using similar techniques, before this conclusion can be verified. Findings from this study might be exclusive to *R. catesbeiana*, and interspecific differences in hormonal-induced oviductal growth might be prevalent among amphibians.

It is interesting to note that oviductal growth in sham frogs was not similar to growth in E_2- and E_2/IGF-1 treated frogs. It was assumed that the sham frogs would exhibit oviductal growth, similar to E_2-treated frogs but greater than that of placebo frogs, because their intact

ovaries would continue to synthesize and secrete E_2 throughout the study. There are several possible explanations for these findings.

It is possible that the implants in E_2- and E_2/IGF-treated frogs contained E_2 concentrations higher than is typically found in *R. catesbeiana*. E_2 doses were determined based on studies of E_2 necessary to elicit oviductal growth in *Xenopus laevis* (Follett and Redshaw, 1967; Redshaw et al., 1968) and in reptiles (Cox, 1994). Thus, these doses might have been comparatively high for *R. catesbeiana*. Unfortunately, prior to this study, there were no published references for physiologically relevant doses of E_2 capable of stimulating oviductal growth in ovariectomized *R. catesbeiana*. Regardless, this explanation still seems less probable than the ones that follow.

Another explanation for relatively low oviductal growth in sham frogs was that they were different from E_2- and E_2/IGF-1-treated frogs with respect to pre-surgery and post-treatment plasma IGF-1 concentrations. In E_2- and E_2/IGF-1-treated frogs, plasma IGF-1 concentrations increased after treatment compared to pre-ovariectomy levels. In sham frogs, however, plasma IGF-1 concentrations after sham-treatment remained similar to pre-surgery concentrations. In E_2- and E_2/IGF-1-treated frogs, the increase in plasma IGF-1 could have stimulated increased IGF-1R and expression in oviductal tissues, making them more sensitive to E_2- and IGF-1-induced growth. As mentioned above, IGF-1R does interact, or exhibit "cross-talk" with the ERα in stimulating oviduct growth (Klotz et al., 2002). There was no change in mean plasma IGF-1 concentrations in sham frogs before and after the experiment. With no change in IGF-1 concentrations, it is possible that oviductal IGF-1R expression also remained unchanged, or

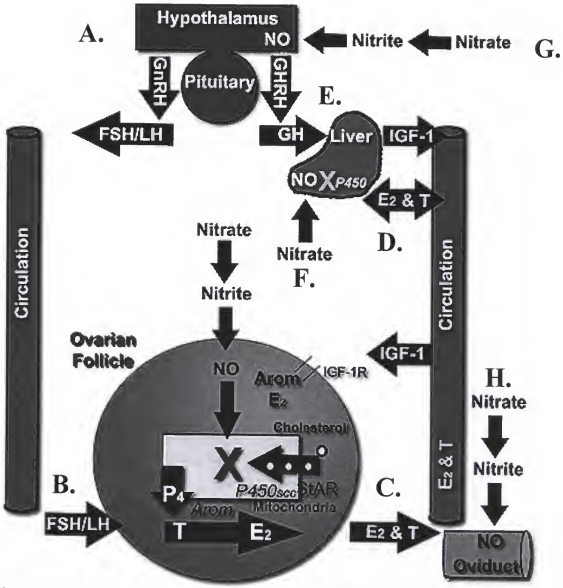

Figure 7-1. Regulation of gonadal steroid synthesis and metabolism, and of hepatic IGF-1 synthesis. A Hypothalamic gonadotropin releasing hormone (GnRH) induces pituitary luteinizing hormone (LH) and follicle stimulating hormone (FSH) secretion. B. LH stimulates testosterone (T) synthesis from progesterone (P_4) precursor. FSH stimulates aromatase enzymes (arom) to convert T into estrogen (E_2). C. Steroids (E_2 & T) enter circulation and can inhibit GHRH and GnRH release, and D. be metabolized and removed from circulation by liver P450 enzymes. E. Hypothalamic growth hormone -releasing hormone (GHRH) stimulates pituitary growth hormone (GH) secretion. GH stimulates liver insulin-like growth factor-1 (IGF-1) synthesis and secretion. F. Nitrate metabolized by the liver can cause NO formation, which can inhibit hepatic P450 steroid metabolizing enzymes, preventing steroid metabolism and clearance from circulation. G. Nitrate and nitrite stimulation formation of hypothalamic NO GHRH release, and pituitary GH, which stimulates the liver to secrete IGF-1 into circulation. stimulating GHRH H. Nitrate and nitrite might inhibit oviduct growth through conversion to nitric oxide (NO), which can induce apoptosis in tissue.

baseline, reducing ERα "cross-talk", and making oviductal tissue comparatively less sensitive to E_2- or IGF-1-induced stimulation of growth (Figure 7-1). Future studies should examine oviduct growth in response to steroid and growth factors hormones and also in response to ERα and IGF-1R expression to better understand their possible interactions.

Lastly, there might have also been an "implant effect" on oviductal growth. With or without hormones, the implant might have elicited oviductal hypertrophy due to an irritation response to a "foreign body" within the abdomen. In summary, much more research is necessary to discern the potential influence of these variables on oviduct growth in *R. catesbeiana*.

It is interesting to report that nitrate exposure of *R. catesbeiana* was associated with decreased oviductal endometrial layer thickness and gland surface-area. It is possible nitrate exposure increased endogenous nitrite levels and nitric oxide (NO) formation in the oviduct. NO is known to induce apoptosis, or programmed cell death. NO can induce apoptosis through inhibition of heme-containing enzymes involved in cell growth (Lincoln et al., 1995). An increase in oviductal NO could also lead to formation of peroxynitrite, a cytotoxic compound that causes cellular damage and inflammation (Bastian et al., 2002).

Nitrate Exposure (*In Vivo* and *In Vitro*): Effects on Steroidogenesis

Nitrate exposure has been reported to inhibit *in vivo* and *in vitro* steroidogenesis in mammals (Panesar and Chan, 2000). Nitrate within the body can be readily converted to nitrite, and visa versa, by endogenous microbial activity and oxidation (Jensen, 1995; Doblander and Lackner, 1996, 1997; Jensen, 2003). Within the liver and other tissues, nitrite is then metabolized into nitric oxide (NO) (Doblander and Lackner, 1996). NO is known to inhibit steroidogenesis by binding to the heme groups of cytochrome P450 (CYP) enzymes (Figure 7-1). The steroid synthetic enzymes include side-chain cleavage (CYP11), 17β-hydroxylase (CYP17), and aromatase (CYP19). Side-chain cleavage is the major rate-limiting step in the steroidogenic pathway (Jensen, 2003). Studies in fish and turtles indicate that similar steroidogenic enzymes

occur in mammalian and non-mammalian vertebrates (Sakai et al., 1992; Takahashi et al., 1993; Jeyasuria et al., 1994; Omura, 1999).

In this dissertation, different responses of frogs were observed with exposure to *in vitro* and *in vivo* nitrate. *Xenopus laevis* exposed to *in vivo* nitrate for seven days exhibited no difference in plasma steroid concentrations among treatments while *ex vivo* ovarian steroid concentrations, synthesized by isolated tissue, were significantly decreased in nitrate-exposed frogs. Similar to *ex vivo* steroidogenesis observed in *X. laevis*, *in vitro* steroidogenesis in *R. grylio* ovaries exhibited decreased steroid synthesis when exposed to nitrate (Chapter 5).

The basis for the normal *in vivo* steroid concentrations but decreased ovarian *ex vivo* steroid concentrations in *X. laevis* remains unclear but likely involves endocrine feedback of the hypothalamic-pituitary-gonadal (HPG) axis (Figure 7-1). Despite the inhibitory effects of nitrate, normal *in vivo* steroid concentrations were likely maintained by compensatory signaling through the HPG axis. If nitrate exposure inhibited ovarian steroid synthesis in *R. catesbeiana*, a decrease in circulating steroids or inhibin could have stimulated the hypothalamus to secrete gonadotropin-releasing hormone (GnRH) and the pituitary to secrete plasma gonadotropin (FSH and LH). FSH and LH would stimulate continued gonadal steroidogenesis to compensate for the initial inhibition, which could have contributed to normal plasma steroid concentrations. Compensatory *in vivo* responses are reported for human patients that develop nitrate tolerance with continuous treatment of organic nitrate like nitroglycerin or isosorbide dinitrate (Gori and Parker, 2002; Parker, 2004). In contrast to the *in vivo* model, *ex vivo* and *in vitro* steroidogenesis involved culture of ovarian tissue that was physically isolated from hypothalamic-pituitary regulation. In isolated ovarian tissue, nitrate-induced inhibition of steroidogenesis could not be counteracted by compensatory release of hypothalamic GnRH and pituitary gonadotropins (Figure 7-1). Plasma gonadotropins and hypothalamic GnRH were not measured in the *in vivo* study of *X. laevis*; thus, this theory cannot be confirmed.

Another possible mechanism for the increased circulating steroid concentrations in response to nitrate could involve regulation at the level of the hypothalamic-pituitary-hepatic (HPH) axis (Figure 7-1). In mammals, plasma steroid concentrations have also been reported to increase with nitrate exposure due to inhibition of hepatic-steroid degradation (Waxman, 1992; Sivapathasundaram et al., 2003). The liver is the main site for nitrate catabolism where large amounts of NO are produced (Lalka et al., 1993). Hepatic NO can inhibit P450 enzymes involved in steroid metabolism and elimination (Waxman, 1992). Minamiyama et al. (2004) reported for rats that administration of organic nitrate decreased hepatic cytochrome P450 activity within 24 hr of exposure. Other xenochemicals also alter the expression of hepatic P450 enzymes involved in steroid metabolism and elimination (Waxman, 1992). Minamiyama et al. (2004) reported for rats that administration of organic nitrate decreased hepatic cytochrome P450 activity within 24 hr of exposure. Other xenochemicals also alter the expression of hepatic P450 enzymes (Waxman, 1999). Therefore, despite a depression of steroid synthesis in the gonad, as seen in *X. laevis* and *R. grylio*, a severe nitrate-induced depression of P450 activity in the liver could have depressed steroid clearance in *R. catesbeiana* and plasma steroid concentrations were actually augmented (Figure 7-1).

Ovarian *ex vivo* steroid inhibition in *X. laevis* was similar to ovarian *in vitro* steroid inhibition in *R. grylio* in that a dose-dependent steroid response curve was not observed with nitrate exposure. The absence of a dose-dependent decrease in steroidogenesis with increasing nitrate exposure in these two studies offers several explanations. For one, the difference between the lowest and highest nitrate concentration might have been insufficient to produce a differential response in steroid synthesis. For *ex vivo* culture of *X. laevis* ovaries, frogs were exposed to nitrate concentrations of 24.75 and 49.50 mg/L NO_3-N, whereas, *in vitro* culture of *R. grylio* ovaries involved nitrate exposure ranging in concentration from 0.17 - 33.00 mg/L NO_3-N. Thus, the lowest and highest nitrate concentrations appeared equally capable of inhibiting steroid synthesis. For *R. grylio*, it is also possible that the steroid-inhibition threshold is actually much

lower than the lowest nitrate (0.17 mg/L) and nitrite (0.20 mg/L) concentration examined. Future studies should include a wider range of nitrate and nitrite concentrations; for example, ranging from 25 μg/L and 1000 mg/L. Reports of amphibians exposed to atrazine have shown that extremely low concentrations (25 μg/L) are capable of inducing a 10-fold decrease in steroid synthesis (Hayes et al., 2002). Similar to the sensitivity of amphibian gonads to disruption by atrazine, ovarian steroidogenesis is apparently sensitive to endocrine disruption by extremely low concentrations of nitrate.

Nitrate Exposure and Plasma IGF-1

The investigations in this dissertation provide the first evidence that nitrate exposure of amphibians to environmentally relevant nitrate concentrations, ranging from 1.65 - 49.50 mg/L, is associated with increased plasma IGF-1 concentrations in *X. laevis* and *R. catesbeiana*. The physiological mechanism regulating nitrate-associated increases in circulating IGF-1 is unclear but a hypothesis has been proposed (Chapter 2).

There is increasing evidence that IGF-1 functions as a regulator of steroid function and secretion (Hammond et al., 1991; Adashi, 1993; Devoto et al., 1999; Driggers and Segars, 2002). Increased circulating IGF-1 concentrations might counteract nitrate-induced steroid inhibition (Figure 7-1). Intraovarian NO formation, in response to nitrate exposure, might increase expression of IGF-1. Increased intraovarian IGF-1 has been shown to increase expression of P450 aromatase enzyme (CYP19) and E_2, and also regulates estrogen-induced growth of reproductive tissues (Daughaday and Rotwein, 1989; Erickson et al., 1989; Monnieaux and Pisselet, 1992; Adashi, 1993; Samaras et al., 1994; Hiney et al., 1996; Olsen et al., 1996; Samaras et al., 1996; Dees et al., 1998). NO also might influence IGF-1 at the level of the hypothalamic-pituitary-hepatic axis. NO participates in the regulation of GH secretion at level of the HPH axis, thereby increasing hepatic IGF-1 synthesis and circulating IGF-1 concentrations (de Caceres et al., 2003; Figure 7-1). NO not only decreases ovarian P450 aromatase enzyme and E_2 expression but also

increases IGF-1 mRNA (Srivastava et al., 1997; Dees et al., 2000). Thus, the increase in circulating IGF-1 concentrations might derive from non-hepatic sources, including the ovaries.

In summary, increasing evidence suggests that steroids should not be the exclusive endpoint for evaluating endocrine-disruption by nitrate. These endpoints should be expanded include steroid receptors, steroidogenic enzymes, growth factors and their receptors, steroid and IGF-1 binding proteins, and hepatic cytochrome P450 enzymes. Understanding the role of nitrate, steroids, and IGF-1 in normal and abnormal reproduction in amphibians and other animals will be largely dependent upon which endpoints are examined.

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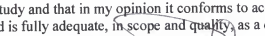
BIOGRAPHICAL SKETCH

Tamatha R. Barbeau was born in Plattsburgh, New York, on 28 September 1970. Many of her summers were spent camping with her family in the NY Adirondacks and in Canada. There she spent much of her time fishing, studying animals, and exploring the northern woodlands. At Canton State University of NY, she studied veterinary science and earned her associates degree and a veterinary technician license in 1990. At Oswego State University of NY, she majored in biology and earned a baccalaureate in 1992. After completing her undergraduate studies, she worked as the head technician in a veterinary hospital for 5 years. A growing desire to work with a wide diversity of animals in their natural environments inspired her to change careers and enter graduate school. She joined the Department of Zoology at the University of Florida in 1997 and, working with Dr. Harvey B. Lillywhite, was awarded a Master of Science degree in 2000.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Louis J. Guillette, Jr., Chair
Distinguished Professor of Zoology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.



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Lauren J. Chapman
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David H. Evans
Professor of Zoology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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This dissertation was submitted to the Graduate Faculty of the Department of Zoology in the College of Liberal Arts and Sciences and to the Graduate School and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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